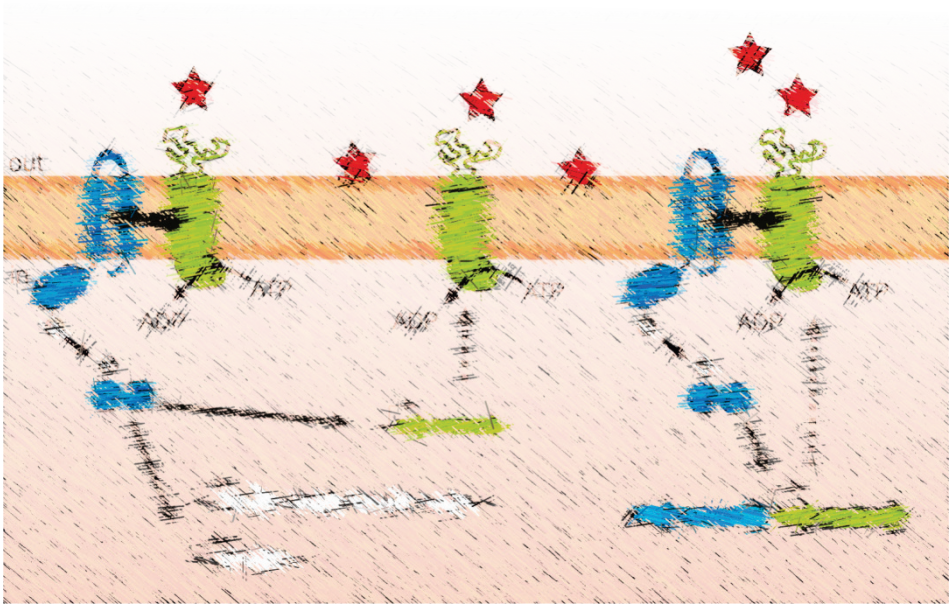
UNIVERSITAT POLITÈCNICA DE VALÈNCIA

IATA

CSIC
Consejo Superior de Investigaciones Científicas

Characterization of Two Component Systems of *Lactobacillus casei* BL23 and their involvement in stress response



PhD Thesis

Ainhoa Revilla Guarinos

October, 2014

Supervisor:
Dr. Manuel Zúñiga Cabrera

UNIVERSIDAD POLITÉCNICA DE VALENCIA
Departamento de Biotecnología

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
Instituto de Agroquímica y Tecnología de Alimentos
Laboratorio de Bacterias Lácticas y Probióticos

Characterization of Two Component Systems of *Lactobacillus casei* BL23 and their involvement in stress response

Dissertation submitted in partial fulfilment of the requirements for obtaining the degree of International Doctor (Ph. D.) in Biotechnology

By
Ainhoa Revilla Guarinos

Supervisor
Manuel Zúñiga Cabrera

February, 2014

El Dr. Manuel Zúñiga Cabrera, Investigador Científico del Consejo Superior de Investigaciones Científicas (CSIC) con destino en el Instituto de Agroquímica y Tecnología de Alimentos (IATA):

CERTIFICA:

Que Dª. **Ainhoa Revilla Guarinos**, Licenciada en Bioquímica por la Universidad Autónoma de Madrid y Postgraduado en Biología Molecular Celular y Génetica, especialidad Bioquímica y Biología Molecular, por la Universidad de Valencia, ha realizado bajo mi dirección el trabajo que, con el título **"Characterization of Two Component Systems of *Lactobacillus casei* BL23 and their involvement in stress response"**, presenta para optar al grado de Doctor Internacional en Biotecnología por la Universidad Politécnica de Valencia.

Y para que así conste a los efectos oportunos, firma el presente certificado en Paterna, a de de 2014.

Dr. Manuel Zúñiga Cabrera

El Dr. Manuel Hernández Pérez, Profesor Titular de la Universidad Politécnica de Valencia:

CERTIFICA:

Que Dª. **Ainhoa Revilla Guarinos**, Licenciada en Bioquímica por la Universidad Autónoma de Madrid y Postgraduado en Biología Molecular Celular y Génetica, especialidad Bioquímica y Biología Molecular, por la Universidad de Valencia, ha realizado bajo su tutela el trabajo que, con el título **"Characterization of Two Component Systems of *Lactobacillus casei* BL23 and their involvement in stress response"**, presenta para optar al grado de Doctor Internacional en Biotecnología por la Universidad Politécnica de Valencia.

Y para que así conste a los efectos oportunos, firma el presente certificado en , a de de 2014.

Dr. Manuel Hernández Pérez

A mis yayos, Arsenio y Pilar.

A mis padres, Tere y Jesús.

A mi hermana Maite,

Tas…

AGRADECIMIENTOS, PARTE 1.

Ha llegado el momento de escribir lo mejor de la tesis, ¡los agradecimientos!

Agradezco al antiguo Ministerio Español de Ciencia e Innovación la asignación de una beca FPI (BES-2008-004527) a Manolo Zúñiga Cabrera, y agradezco mucho más a Manolo que me eligiera a mí como destinataria de esa beca. Gracias a esa FPI la realización de esta tesis ha sido posible. Y gracias a esa beca FPI mi vida cambió no sólo de ubicación física (de Madrid a Valencia), sino en cierto sentido, también de rumbo. Agradezco también al Instituto Danone la concesión de una Beca de Investigación, la cual le ha dado el empujón final a esta tesis. Gracias a Manolo por ser mi director de tesis, sin él nada de esto habría sido posible. Gracias también a Manuel Hernández por su labor como tutor de esta tesis en la Universidad Politécnica de Valencia.

GRACIAS con mayúscula a todos los componentes actuales y pasados, del grupo de Bacterias Lácticas y Probióticos del IATA. Pese a la disparidad de caracteres y líneas de investigación, se mantiene un sentimiento de grupo muy acogedor y la sensación de que el conjunto te apoya es reconfortante.

GRACIAS, GRACIAS, GRACIAS a mi maestra en el laboratorio 007, Cristina Alcántara Baena. De ti he aprendido no sólo una manera de hacer Ciencia sino de ver la Vida. He de dar las gracias también a mi primer maestro antes de mi llegada al laboratorio 007, Edel Raidel, ya que me ayudó a asentar los cimientos de mi actitud científica sobre los cuales seguí construyendo.

Gracias a Vicente Monedero por tener siempre su puerta abierta y sus silla de invitados disponible para oírme, aconsejarme y tranquilizarme.

Son tantas las personas de las cuales he aprendido profesionalmente hablando, que no voy a mencionarlas por miedo a olvidarme a alguna. A todas aquellas que me ayudaron, y también a los que no me ayudaron, muchísimas gracias. Porque de todos he aprendido algo.

GRACIAS con mayúsculas a todos los AMIGOS que he encontrado desde mi llegada a Valencia y durante mis estancias en München, y a aquellas

personas que aunque pasaron tan sólo “un momento” por mi vida, marcaron puntos de inflexión. Tampoco los voy a nombrar porque no hace falta, porque todos ellos ya saben quiénes son y que están en mi corazón. Gracias por haber contribuido a que estos años de tesis hayan sido mucho más, gracias por llenar este tiempo de VIDA.

Gracias a www.phdcomics.com por recordarme muchos días que no estaba sola en mi *thesis repulsor field* de mi *graduate space-time*.

Agradecimiento especial a Concha, por enseñarme a ver la luz al final del túnel.

Y finalmente, INFINITAS GRACIAS a los más importantes, a mis incondicionales: Mi Familia. Gracias a mis padres, gracias a mi hermana y gracias a mis yayos, porque sé que estarán siempre ahí independientemente “del tiempo” que haga. Gracias por tanto amor. Y gracias a la abuela que me apoya en la distancia comiendo *danones* ;-).

Cuando tengas que elegir entre dos caminos,
pregúntate cuál de ellos tiene corazón.
Quien elige el camino del corazón no se equivoca nunca.

Popol-Vuh

ACKNOWLEDGMENTS, PART 2

I would like to thanks Prof. Dr. Thorsten Mascher for embracing me in his lab at the Ludwig Maximilians Universität of München, where I spend a total of ten months during my two short stays. I would also like to thanks Dr. Susanne Gebhard. Thank you very much to both of you, and to all the people from your labs. You taught me another way of doing Science and made me feel that I was at Home.

Thanks also to all my friends from München, whose names I am not going to write (similarly to what I did with my Spanish friends) because they also know who they are and that they are in my heart. They know that since I have been there, my heart has been divided between two "Homes". Thanks also to all the people that helped me during my time there.

I am finishing these acknowledgements with the advice a *Philosopher* - Scientist friend once gave to me.

A life philosophy.

"I do not find your ambivalence towards your success as surprising as you might think...Only very simple minded people can fully and without any reservations enjoy a victory. And you just expressed what is a "simple" (but not so simple to see!) fact: that anything in life, good or bad, is a double-bladed sword and a transient thing. And as much as you are willing and able to see the single ray of light even in a dark place, so will you never just see the sunny side in a good turning of tides. As tides come, but also go, waves that carry you eventually tend to break. The art in life is to surf them as long as they can possibly carry and enjoy the ride, knowing that they will eventually break. But also resting assured that - when they do - the next wave is already coming in. So Ainhoa: stay afloat, ride the wave, feel the wind in your face! This is what life is about."

Thank you.

ABSTRACT

Lactobacillus casei is a species naturally found in the gastrointestinal and genital tracts of animals and humans. It has been traditionally employed in the production of fermented foods and recent studies have demonstrated the probiotic properties of certain strains of this species, as *L. casei* BL23, the strain subject of the present study.

Probiotics are defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host. In order to exert their beneficial effect, they must reach the intestine in sufficient amounts and in a viable state. Thus, they must be able to survive to the variety of adverse conditions - stress - encountered during industrial food processing and during their transit through the gastrointestinal tract. Among these stress conditions are the presence of antimicrobial peptides (used as food preservatives, produced by the innate immune system of the host or produced by other microorganisms present in the intestinal microbiota) and acid stress (during industrial processing or during passage through the stomach). Signaling systems are critical to detect adverse changes in the environmental conditions and to activate specific responses to counteract them.

Among these signaling mechanisms, the signal transduction Two Component Systems (TCS) play a major role in bacteria. They typically consist of an intramembrane sensor protein, histidine kinase, and a response regulator which controls the expression of specific genes involved in the adaptive response to the stress condition that activated the system. TCS have been extensively studied in model microorganisms like *Escherichia coli* and *Bacillus subtilis*, as well as in other pathogen microorganisms like *Staphylococcus aureus* where they have a major role during the processes of invasion and colonization of the host. Probiotic bacteria must face essentially the same environmental conditions as pathogens do, however, the information about the signal transduction mechanisms operating in probiotic bacteria like *L. casei* is scarcer. This work aims to gain insight into the role of TCS in the physiology and stress response of the probiotic microorganism *L. casei*.

The investigations carried out during this PhD thesis showed that 17 TCS were encoded in *L. casei* BL23 genome. Mutants defective in the 17 response regulators of each of the TCS were obtained and they were phenotypically characterized. Our results highlighted the importance of some of these TCS in the general physiology as well as in the stress response of this strain.

A deeper study was performed in the response to stress by antimicrobial peptides. The results showed that TCS09 and TCS12, belonging to the BceRS-like TCS group which have been described to be involved in the antibiotic stress response in other microorganisms, are also involved in the antimicrobial peptide stress response of *L. casei* BL23. Furthermore, our results have shown that TCS12 is controlling systems involved in maintenance of cell surface properties: Dlt system and MprF protein. Our work proves that a correct functionality of the Dlt system is crucial in the maintenance of the barrier properties of the cell wall of *L. casei* BL23, which is determinant in its ability to survive in acid media.

This work has led to an increased understanding not only of the mechanisms regulating signal transduction, but also of the specific response mechanisms that allow *L. casei* BL23 to adapt its physiology to different environmental conditions.

RESUMEN

Lactobacillus casei es una especie presente de forma natural en el tracto gastrointestinal y genital de animales y humanos. Ha sido empleada tradicionalmente en la producción de alimentos fermentados y estudios recientes han demostrado las propiedades probióticas de algunas cepas de esta especie, como *L. casei* BL23, la cepa objeto del presente trabajo.

Se definen como probióticos los organismos vivos que ingeridos en cantidades adecuadas tienen un efecto saludable sobre el huésped. Para poder ejercer su efecto beneficioso es necesario por tanto que alcancen el intestino en cantidad suficiente y en un estado viable. Para ello, deben poder sobrevivir en las distintas condiciones adversas –estrés– que encuentran durante el procesado industrial de los alimentos y durante su tránsito por el tracto gastrointestinal. Entre estas condiciones de estrés se encuentran la presencia de péptidos antimicrobianos (usados como conservantes alimenticios, producidos por el sistema inmune innato del huésped, o producidos por otros microorganismos presentes en la microbiota intestinal) y el estrés ácido (durante el procesado industrial o durante el paso por el estómago). Los mecanismos de transducción de señal son fundamentales en este proceso ya que perciben un determinado estímulo –estrés– ambiental y activan una respuesta específica para hacer frente a ese estímulo.

Entre estos mecanismos de transducción de señal se encuentran los sistemas de dos componentes (TCS) constituidos por una proteína sensora intramembranal, histidina quinasa, y un regulador de respuesta que controla la expresión de genes concretos implicados en la respuesta adaptativa a la condición de estrés. Los sistemas de dos componentes han sido ampliamente estudiados en microorganismos modelo como *Escherichia coli* y *Bacillus subtilis*, así como en otros microorganismos patógenos como *Staphylococcus aureus* donde juegan un papel fundamental en el proceso de invasión y colonización del huésped. Las bacterias probióticas deben hacer frente esencialmente a las mismas condiciones ambientales que los patógenos, sin embargo, la información sobre los sistemas de transducción de señal que operan en probióticos como *L. casei* es mucho menor. Este trabajo pretende profundizar en la posible implicación de los sistemas de dos componentes en la

fisiología y la capacidad de respuesta a estrés del microorganismo probiótico *Lactobacillus casei.*

La investigación llevada a cabo en esta tesis mostró la presencia de 17 sistemas de dos componentes codificados en el genoma de BL23. Se obtuvieron mutantes en los 17 sistemas, que se caracterizaron fenotípicamente. Nuestros resultados evidencian la importancia de algunos de estos sistemas en la fisiología general y en la capacidad de respuesta a estrés ambiental de esta cepa.

Se realizó un estudio más amplio de la respuesta a estrés por péptidos antimicrobianos y se demostró que TCS09 y TCS12, pertenecientes al grupo BceRS previamente caracterizado en respuesta a estrés antimicrobiano en otros microorganismos, juegan un papel fundamental también en la respuesta de resistencia a péptidos antimicrobianos de *L. casei* BL23. Más aún, los resultados obtenidos muestran que TCS12 controla sistemas implicados en el mantenimiento de las propiedades de superficie celular: sistema Dlt y proteína MprF. Nuestro trabajo evidencia que un correcto funcionamiento del sistema Dlt es crucial en el mantenimiento de las propiedades barrera de la pared celular de *L. casei* BL23, lo que determina también su supervivencia en medio ácido.

Este trabajo ha supuesto un incremento de conocimiento de los mecanismos de transducción de señal reguladores y de los mecanismos concretos de respuesta, que permiten a *L. casei* BL23 adaptar su fisiología a las distintas condiciones ambientales.

RESUM

Lactobacillus casei és una espècie present de forma natural en el tracte gastrointestinal i genital d'animals i d'humans. Ha estat emprada tradicionalment en la producció d'aliments fermentats i estudis recents han demostrat les propietats probiòtiques d'algunes soques d'aquesta espècie, com *L. casei* BL23, la soca objecte del present treball.

Els probiòtics es defineixen com els organismes vius que, ingerits en quantitats adequades, tenen un efecte saludable sobre l'hoste. Per a poder exercir el seu efecte beneficiós és necessari per tant que arribin a l'intestí en quantitat suficient i en un estat viable. Per això, han de poder sobreviure en les diferents condicions adverses - estrès- que troben durant el processat industrial dels aliments i durant el seu trànsit pel tracte gastrointestinal. Entre aquestes condicions d'estrès es troben la presència de pèptids antimicrobians (utilitzats com a conservants alimentaris , produïts pel sistema immune innat de l'hoste , o produïts per altres microorganismes presents en la microbiota intestinal) i l'estrès àcid (durant el processat industrial o durant el pas per l'estómac). Els mecanismes de transducció de senyal són fonamentals en aquest procés ja que perceben un determinat estímul -estrès ambiental i activen una resposta específica per fer front a aquest estímul.

Entre aquests mecanismes de transducció de senyal es troben els sistemes de dos components (TCS) constituïts per una proteïna sensora intramembranal, histidina quinasa, i un regulador de resposta que controla l'expressió de gens concrets implicats en la resposta adaptativa a la condició d'estrès. Els sistemes de dos components han estat àmpliament estudiats en microorganismes model com *Escherichia coli* i *Bacillus subtilis*, així com en altres microorganismes patògens com *Staphylococcus aureus* on juguen un paper fonamental en el procés d'invasió i colonització de l'hoste. Els bacteris probiòtics han de fer front essencialment a les mateixes condicions ambientals que els patògens, no obstant, la informació publicada sobre els sistemes de transducció de senyal que operen a probiòtics com *L. casei* és substancialment menor. Aquest treball pretén aprofundir en la possible implicació dels sistemes de dos components en la fisiologia i en la capacitat de resposta a l'estrès del microorganisme probiòtic *Lactobacillus casei*.

La investigació portada a terme en aquesta tesi va determinar la presència de 17 sistemes de dos components codificats en el genoma d'BL23. Es van obtenir mutants d'els 17 sistemes, els quals es van caracteritzar fenotípicament. Els nostres resultats evidencien la importància d'alguns d'aquests sistemes en la fisiologia general i en la capacitat de resposta a estrès ambiental d'aquesta soca.

A més es va realitzar un estudi més ampli de la resposta a estrès per pèptids antimicrobians i es va demostrar que els TCS09 i TCS12, pertanyents al grup BceRS prèviament caracteritzat en resposta a estrès antimicrobià en altres microorganismes, també juguen un paper fonamental en la resposta de resistència a pèptids antimicrobians de *L. casei* BL23. Fins i tot, els resultats obtinguts mostren que el TCS12 controla sistemes implicats en el manteniment de les propietats de superfície cel · lular: el sistema Dlt i la proteïna MprF. El nostre treball evidencia que un correcte funcionament del sistema Dlt és crucial per al manteniment de les propietats barrera de la paret cel · lular de *L. casei* BL23, el que determina també la seva supervivència en medi àcid.

Aquest treball ha suposat una ampliació en el coneixement dels mecanismes de transducció de senyal reguladors i dels mecanismes concrets de resposta , que permeten a *L. casei*BL23 adaptar la seva fisiologia a les diferents condicions ambientals.

TABLE OF CONTENTS

ABSTRACT XIII

RESUMEN XV

RESUM XVII

INDEX OF TABLES XXIV

INDEX OF FIGURES XXVI

ABBREVIATIONS XXX

INTRODUCTION

1. Life and habitats in the gastrointestinal tract..... 3

1.1. Different niches of the GIT determine the composition of the microbiota..... 4

1.2. The composition of the human GIT microbiota..... 6

2. Lactic acid bacteria. 9

2.1. Definition and classification..... 9

2.2. *Bifidobacteriales* 9

2.3. *Lactobacillales*..... 11

2.3.1.The genus *Lactobacillus*..... 13

2.4. Probiotics..... 14

3. Cell surface of lactobacillus..... 17

3.1. The cell wall of *Lactobacillus*..... 19

3.1.1. Peptidoglycan..... 19

3.1.2. Teichoic acids..... 22

3.1.2.1. Dlt system..... 23

3.1.3. Cell wall polisaccharides..... 25

3.1.4. Cell surface proteins..... 25

3.1.5. Pili and flagella..... 26

3.2. The cell membrane..... 26

3.2.1. MprF protein..... 28

3.2.2. ATP binding cassette (ABC) transporters 29
4. Methods for assessing bacterial cell surface charge properties 30
4.1. Binding assays with charged molecules..... 30
4.2. Electrophoretic mobility determinations 31
5. Stress response 34
5.1. Low pH response 35
Adaptive acid tolerance response (ATR)..... 36
Physiological changes induced under acid stress 37
5.1.1. Changes in cell surface properties..... 37
5.1.2. DNA and protein repair mechanisms..... 39
5.1.3. Proton pumps..... 39
5.1.4. Production of basic compounds 40
5.1.5. Alteration of metabolic pathways 41
5.2. Responses to stress by antimicrobial peptides (AMPs)..... 41
5.2.1. Removal of the peptides from their site of action by ABC transporters..... 45
5.2.1.1. LanFEG and BcrAB type transporters..... 48
5.2.1.2. BceAB like transporters..... 51
5.2.2. Modification of cell surface properties..... 51
5.2.3. Biofilms as antibiotic induced resistance mechanisms..... 55
6. Signal transduction: Two Component Systems (TCS)..... 56
6.1. Bce like TCS..... 59
7. TCS in *Lactobacillus casei* BL23..... 67

References..... 70

OBJECTIVES

Objectives..... 97

RESULTS

CHAPTER 1 PART I:
Influence of Two-Component Signal Transduction Systems of *Lactobacillus casei* BL23 on Tolerance to Stress Conditions...... 101

Abstract 102

Introduction 103
Materials & methods 104
Results & Discussion 106
Acknowledgments 110
Supplemental material 111
References 123

CHAPTER 1 PART 2:
Influence on the general physiology of *Lactobacillus casei* BL23 of Two-Component Signal Transduction Systems 127

Abstract 128
Introduction 129
Materials & methods 131
Bacterial strains and growth conditions..... 131
Growth in skim milk..... 131
Phenotypic characterization of TCS12 mutant strains..... 131
Microscopic characterization..... 133
Results 134
Growth in skim milk..... 134
Growth of TC12 strain in buffered media 135
pH homeostasis studies with BL23 and ΔRR12 strains..... 137
Microscopic characterization of ΔRR12 strain..... 138
Discussion 142
References 145

CHAPTER 2:
Characterization of a regulatory network of peptide antibiotic detoxification modules in *Lactobacillus casei* BL23..... 149

Abstract 150
Introduction..... 151
Materials & methods..... 155
Bacterial strains, plasmids, and growth conditions..... 155
Comparative genomics and motif-based search for putative *Bce*-like response regulator binding sites..... 155
Construction of mutants..... 157
Cytochrome *c* binding assay..... 158
Phenotypic characterization of *L. casei* BL23 and mutants..... 158

Reverse transcription and quantitative real-time PCR (qRT-PCR)..... 159
Results..... 160
L. casei BL23 encodes two BceRS-like TCS and three BceAB-like ABC transporters in its genome..... 160
Inactivation of Bce-like TCS systems or their cognate ABC transporters results in increased sensitivity to antimicrobial compounds..... 160
Transcriptional response of *L. casei* BL23 and derived strains to nisin..... 162
Identification of putative target promoters of RR09 and RR12 and verification of the predictions by qRT-PCR..... 166
The pleiotropic phenotype of module 12-defective strains is due to a low expression of the dlt operon..... 169
Discussion..... 174
Acknowledgments..... 178
Supplemental material 179
References 182

CHAPTER 3:
Characterization of the response to low pH of *Lactobacillus casei* ΔRR12, a mutant strain with low D-alanylation activity and sensitivity to low pH..... 191

Abstract..... 192
Introduction..... 193
Materials & methods..... 196
Bacterial strains and growth conditions 196
Determination of cell membrane fatty acid composition..... 196
Two-dimensional SDS-PAGE analysis of the response of *L. casei* BL23 and ΔRR12 to acid challenge..... 197
Results..... 199
The mutant strain ΔRR12 can induce a growth phase-dependent acid tolerance response (ATR)..... 199
Variation of the cell membrane fatty acid composition 201
Proteomic analysis of the response of L. casei BL23 and ΔRR12 to sublethal acidic pH exposition..... 204
Discussion..... 209
Acknowledgments 215
Supplemental material 216

References 219

GENERAL DISCUSSION & CONCLUSIONS

General discussion 227
Final conclusions 236
Conclusiones finales 237
References 238

INDEX OF TABLES

Introduction

Table 1. Dominant types of microbes either allochthonous or autochthonous in the major habitats of the human gastrointestinal tract 8

Table 2: Lactic acid bacteria taxonomy classification according to the National Center for Biotechnology Information 10

Table 3: Summary of ABC transporters main characteristics 46

Chapter 1 Part I

Table 1. Results of the growth assays carried out with *L. casei* BL23 and selected TCS defective mutants under different growth conditions 106

Table S1. Histidine kinase and response regulators identified in *L. casei* genomes 111

Table S2. Bacterial strains and plasmids used in this study 112

Table S3. Oligonucleotides used in this study 114

Table S4. Maximum specific growth rates and ΔOD_{595} of different *L. casei* BL23 mutants grown in MRS broth in microplates 116

Table S5 A. Maximal growth rate (μ_{max}) values and pairwise two way ANOVA under different stress conditions 117

Table S5 B. Increment in optical density (Δ O.D.) values and pairwise two way ANOVA at different stress conditions 118

Table S6. MIC values of *L. casei* BL23 and TCS-defective derivative mutants 119

Chapter 1 Part II

Table 1. Bacterial strains used in this study 132

Table 2. Morphologic characterization of stationary phase cultures of BL23 and ΔRR12 strains grown in reference conditions (MRS) and in buffered media (MRS + 0.1 M K_2HPO_4) 139

Chapter 2

Table 1. Bacterial strains and plasmids used in this study 156

Table 2. MIC values of AMPs against *L. casei* BL23 and derivative strains 162

Table S1. Primers used in this study 179

Table S2. BceRS-BceAB like resistance modules identified in *L. casei* BL23 181

Chapter 3

Table 1. Growth-phase dependent ATR induction in *L. casei* BL23 and ΔRR12. ATR is represented by the percentage of survival of the acid challenged cells (incubation for 45 minutes at 37ºC in MRS adjusted to pH 2.5 with HCl) relative to the control non-acid challenged cells. OD_{595} and pH of the cultures at the time of acid challenge is indicated..... 200

Table 2. Variation of fatty acid composition of *L. casei* BL23 and ΔRR12 during growth. Fatty acids: myristic (C14), myristoleic (C14 c9), palmitic (C16), palmitoleic (C16 c9), cis-11-hexadecenoic (C16 c11), stearic (C18), oleic (C18 c9), vaccenic (C18 c11), and thecyclopropanes dihydrosterculic (C19 cyc9) and lactobacillic acid (C19 cyc11) 202

Table 3. Differential spots detected in the proteomic analysis of the membrane protein fractions of *L. casei* BL23 and ΔRR12 mutant cells after an acid challenge at pH 4.4 for 45 minutes 206

Table S1. Characteristics of the identified protein spots differentially expressed of *L. casei* BL23 and ΔRR12 mutant cells after an acid challenge at pH 4.4 for 45 minutes 216

INDEX OF FIGURES

Introduction

Figure 1. Different niches of the gastrointestinal tract (GIT) determine the composition of the microbiota. **A**, The major sections of the GIT. **B**, Schematic representation of the human intestine indicating its different regions, their most relevant physicochemical characteristics and the overall sizes of the residing bacterial populations. **C**, pH levels moving from the stomach to the distal gut and biomass levels 5

Figure 2. Glycolytic routes of lactic acid bacteria (only the more relevant reactions are showed). **A**,"bifidus shunt"; **B**, heterofermentative and **C**, homofermentative routes of *Lactobacillales* 12

Figure 3. Schematic representation of *Lactobacillus* cell surface 18

Figure 4. Schematic representation of peptidoglycan biosynthesis and its inhibition by antibiotics 21

Figure 5. Schematic representation of the Double Layer. The diagram shows the ionic concentration and potential difference as a function of distance from the charged surface of a particle suspended in a dispersion medium. The distribution of positive and negative ions around the charged colloid is shown 32

Figure 6. Overview of some of the mechanisms identified in enhancing acid tolerance of bacteria 38

Figure 7. Structural and compositional diversity of antimicrobial peptides. Schematic representations of the structures of nisin, bacitracin, ramoplanin and protegrin 43

Figure 8. Mode of action of nisin 44

Figure 9. Schematic representation of the postulated models of action of LanFEG and BcrAB transporters conferring CAMP resistance. **1)** Transport assisted by NukH type immunity proteins. **2)** Binding of CAMPs by NisI-type immunity proteins. **3)** Hydrophobic vacuum-cleaner model of efflux mechanism. **4)** CAMPs (bacitracin) bind to the pyrophosphate group of

UPP preventing its dephosphorylation by undecaprenyl pyrophosphate phosphatase (UppP) 49

Figure 10. Modification of the bacterial cell envelope components involved in CAMP resistance. D-alanylation of teichoic acids and L-lysynilation of phosphatidylglycerol 52

Figure 11. Models of the effect of changes in the bacterial cell surface in CAMPs resistance. **A,** cell envelope in the absence of D-alanylation of TAs and L-lysinilation of membrane phospholipids. **B,** electrostatic hindrance model for CAMP resistance. **C,** electrostatic and steric hindrance model 54

Figure 12. Schematic representation of the postulated models of BceRSAB-type modules conferring CAMP resistance. **1)** Sensing, **2)** resistance and **3)** dual function BceAB-like transport systems 60

Figure 13. Schematic representation of clusters containing genes encoding Two Component Systems in *Lactobacillus casei* BL23 68

Chapter 1 Part I

Figure 1. Growth of *L. casei* BL23 and TCS-defective mutants under different conditions (only strains that displayed significant differences with strain BL23 are shown) 107

Figure S1. Growth of BL23 and TC01-defective mutants under different conditions 120

Figure S2 A. Growth of BL23 and TCS06-defective mutants under different conditions 121

Figure S2 B. Growth of BL23 and TCS12-defective mutants under different conditions 122

Chapter 1 Part II

Figure 1 A. Growth in skim milk. Viable cell counting of *L. casei* BL23 and derivative mutants after 48 h of growth in skim milk 134

Figure 1 B. Growth in skim milk. pH variation during growth in skim milk (only strain BL23 and selected mutants are shown) 135

Figure 2. Effect of buffered media in growth. Growth of BL23 and mutant TC12 in MRS supplemented with 0.1 M **(A)** or 0.2 M **(B)** potassium phosphate. **(C)** Viable cell counting and final pH determined after 24 h of growth in reference conditions and buffered media 136

Figure 3. pH homeostasis studies. (A) *L. casei* BL23 and **(B)** ΔRR12, recovery of growth in reference conditions upon one hour of acid exposure. **(C)** Survival rate after the acid challenge 138

Figure 4 A. Morphologic characterization of stationary phase cultures of BL23 and ΔRR12 strains grown in reference conditions (MRS) 140

Figure 4 B. Morphologic characterization of stationary phase cultures of BL23 and ΔRR12 strains grown in buffered media (MRS + 0.1 M K_2HPO_4) 141

Chapter 2

Figure 1. Schematic representation of the signaling network of BceRS/BceAB-like sensory and resistance modules of *L. casei* BL23 154

Figure 2. Relative transcript levels of *L. casei* BL23 *bceRS* and *bceAB* homologous genes after nisin induction compared to their expression in the reference condition 163

Figure 3. Expression of *bce*-like genes in mutant backgrounds. Relative transcript levels of *bceRS* and *bceAB* homologous genes in *L. casei* BL23 derived strains are shown compared to the parental strain **(A)** in the absence of nisin and **(B)** 10 minutes after nisin addition (22.5 ng ml-1). **(C)** Relative transcript levels of the same genes in *L. casei* BL23 and derivative strains 10 minutes after nisin addition (22.5 ng ml^{-1}), compared to the same strains in the absence of nisin 165

Figure 4. (A) Consensus binding sequence for BceR-like regulators. **(B)** Sequence alignment of putative BceR like target sequences identified in *L. casei* BL23 complete genome by motif based search using consensus sequence shown in (A) 167

Figure 5 A and B. Expression of *dlt* operon and *mprF* in mutant backgrounds. Relative transcript levels of genes *dltA* and *mprF* in *L.casei* BL23 derivative strains compared to the parental strain **(A)** in the absence of nisin and **(B)** 10 minutes after nisin addition (22.5 ng ml^{-1}) 168

Figure 5 C. Expression of *dlt* operon and *mprF* in mutant backgrounds. **(C)** Relative transcript levels of *L. casei* BL23 and derivative strains after nisin addition (* 22.5 ng ml^{-1}; ** 750 ng ml^{-1}) compared to the same strains in the absence of nisin 169

Figure 6 A. Growth of *L. casei* BL23 and strains ΔRR12, Per12, DLT and MPRF under reference conditions 170

Figure 6 B, C, D and E. Growth of *L. casei* BL23 and strains ΔRR12, Per12, DLT and MPRF under different conditions. **(B)** In the presence of 0.5 % bile. **(C)** 42 ºC. **(D)** Medium adjusted to pH 4. **(E)** Buffered media (MRS supplemented with 0.1 M phosphate) 171

Figure 7. Cytochrome *c* binding assay. Binding is expressed as a percentage of bound cytochrome *c* to stationary phase cells of strain BL23 and derivatives after incubation with 150 µg ml^{-1} cytochrome *c* for 10 minutes at room temperature 172

Chapter 3

Figure 1. Variation of OD_{595} and medium pH during growth of *L. casei* BL23 and its derivative strain ΔRR12 in MRS at 37 ° C. The vertical solid and dotted lines indicate the sampling points at which ATR induction was detected for strains BL23 and ΔRR12, respectively 201

Figure 2. Variation of the percentage of fatty acid classes of strains BL23 and ΔRR12 during growth 203

Figure 3. Silver-stained 2D electrophoresis gels of cell membrane fraction protein extracted from *L. casei* BL23 or ΔRR12 cells diluted in MRS (pH 6.8) or acid MRS (pH 4.4). **A,** BL23 pH 6.8; **B,** ΔRR12 pH 6.8; **C,** BL23 pH 4.4; **D,** ΔRR12 pH 4.4..... 205

ABBREVIATIONS

aa: amino acid
ABC transporter: ATP-binding cassette transporter
AMPs: antimicrobial peptides
ATR: acid tolerance response
B2H: bacterial two hybrid
CAMPs: cationic antimicrobial peptides
CD: Crohn's disease
CESR: cell envelope stress responses
CFAs : cyclopropane fatty acids
Cyt *c*: cytochrome *c*
ECD: extracytoplasmic domain
ECF: extracytoplasmic function
EFSA: European Food Safety Authority
ELS: electrophoretic light scattering
FA: fatty acid
FDA: American Food and Drug Administration
GIT: gastrointestinal tract
GRAS: Generally Recognized as Safe
HK: histidine kinase
IBD: inflammatory bowel diseases
IC_{50}: 50%inhibitory concentration
IM-HK: intramembrane-sensing histidine kinase
LAB: lactic acid bacteria of the order *Lactobacillales*
LI: large intestine
LTA: lipoteichoic acid
Lys-PGly: lysyl-phosphatidylglycerol
MIC: minimal inhibitory concentration
μ_{max}: maximal growth rate
MprF: multiple peptide resistance factor
MSD: membrane spanning domain
NAG: N-acetylglucosamine
NAM: N-acetyl muramic acid
NBD: nucleotide-binding domain

OD_{595}: optical density at 595 nm
PBP: penicillin-binding protein
Pep7E: ABC transporter of the peptide-7 exporter family
PGly: phosphatidylglycerol
pH_{in}: cytoplasmic pH
pH_{out}: external pH
ΔpH: pH gradient
PMF: proton motive force
QPS: Qualified Presumption of Safety
qRT-PCR: quantitative real-time PCR
RR: response regulator
SI: small intestine
TA: teichoic acid
TCS: two-component system
TMD: transmembrane domain
UC: ulcerative colitis
UP: undecaprenyl-phosphate
UPP: undecaprenyl-pyrophosphate
UppP: undecaprenyl pyrophosphate phosphatase
v/v: volume/volume
w/v: weigh/volume
WTA: wall teichoic acids

INTRODUCTION

Slow down.

Calm down.

Don´t worry.

Don´t hurry.

Trust the process.

Alexandra Stoddard

1. LIFE AND HABITATS IN THE GASTROINTESTINAL TRACT

It has been generally accepted until recently that humans were born with a sterile gastrointestinal tract (GIT) whose colonization started at the moment of birth: during the first hours of life the mother´s vaginal and fecal microbiomes were the main source of inoculum. However, new evidences are emerging that indicate that the *in utero* environment may not be sterile as originally thought, since bacteria such as *Enterococcus faecalis*, *Staphylococcus epidermidis* and *Escherichia coli* have been isolated from the meconium (earliest feces) of healthy neonates (Jiménez *et al.*, 2008).

The gastrointestinal bacterial communities are dynamic systems whose composition changes during the lifetime of the host, mainly depending of changes in diet and life events (aging, illnesses, antibiotic treatments…). Notwithstanding, adult individuals usually host a relatively stable microbiota (Koenig *et al.*, 2011; Nicholson *et al.*, 2012). Host genetic factors mainly related with immunity, as well as environmental and stochastic factors, affect community composition (Bourlioux *et al.*, 2003; Benson *et al.*, 2010). Furthermore, the order in which bacteria arrive to the GIT also affects community composition since microorganisms modify their environment consequently shaping the physicochemical properties of the habitat for other microorganisms (Walter and Ley, 2011). The GIT contains one of the most complex ecosystems studied to date, and the interest to know its composition and possible implications in health and illness as well as its contribution to anatomy and physiology of the host, has been steadily growing (Bourlioux *et al.*, 2003; Sanders, 2003; Food and Agriculture Organization of the United Nations & World Health Organization, 2006; Clarke *et al.*, 2012; Martin *et al.*, 2013; Robles Alonso and Guarner, 2013; Sanders *et al.*, 2013).

1.1. Different niches of the GIT determine the composition of the microbiota

In order to facilitate the digestive process, the human digestive tract is partitioned into several compartments (Figure 1, A and B). As a consequence, a diversity of different niches are generated that result in an unequal distribution of microbes. The digestion of the ingested food starts in the mouth during mastication in the presence of digestive enzymes (such as amylase and lipase) secreted by the salivary glands. Through the esophagus, the digestive bolus reaches the stomach, where the acidic pH favors the action of the proteases. Digestion continues in the small intestine (SI) where proteases, lipases and amylase are added from the pancreas and liver. Simple sugars, amino acids and fatty acids (FAs) are absorbed in the SI. The microbial biomass is kept quite low in the proximal parts of the human gut, with 10^{2-3} cells ml^{-1} in the stomach rising to 10^{8} cells ml^{-1} in the ileum (the distal portion of the SI) (Figure 1). The food components that have not been digested in the proximal part of the GIT (like fiber, resistant starch, some peptides and lipids) pass into the large intestine (LI) through the ileocecal valve. Here, food components that could not be digested by the human enzymes, like nondigestible carbohydrates from plant and animal sources can be utilized by the resident microbiota that reaches here the highest densities in the whole GIT (10^{11} cells ml^{-1} in the colon) (Walter and Ley, 2011).

Different factors contribute to maintain the uneven distribution of microbial biomass through the human gut (Figure 1, B and C). In the stomach and SI microbial population is kept low by: the low pH of gastric contents (the pH in the stomach varies from 1.5 to 5), the rapid luminal flow, the presence of bile salts in the duodenum (strongly bactericidal), and components of the host immune system (like immunoglobulin A and antimicrobial peptides). On the contrary, in the LI the less acidic pH (pH in the colon varies from 5 to 7), the slower peristalsis, lower concentrations of bile salts and the weaker immune responses, allow the presence of bigger bacterial populations (Walter and Ley, 2011).

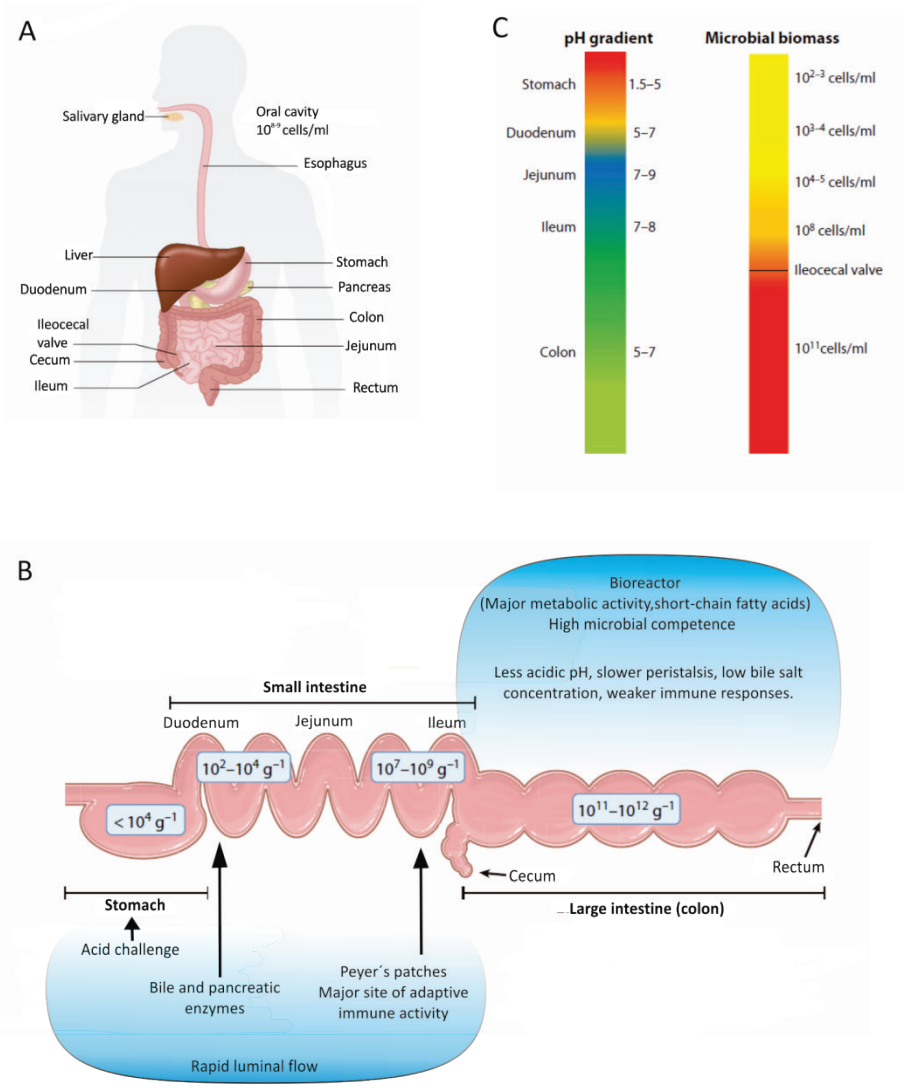


Figure 1. Different niches of the gastrointestinal tract (GIT) determine the composition of the microbiota. A, The major sections of the GIT. B, Schematic representation of the human intestine indicating its different regions, their most relevant physicochemical characteristics and the overall sizes of the residing bacterial populations. C, pH levels moving from the stomach to the distal gut (*left*) and biomass levels (*right*). A and C, from *The Human Gut Microbiome: Ecology and Recent Evolutionary Changes* (Walter and Ley, 2011); B, adapted from *Probiotic and Gut Lactobacilli and Bifidobacteria: Molecular Approaches to Study Diversity and Activity* (Kleerebezem and Vaughan, 2009).

The gastric acid barrier regulates the composition and population of lower intestinal microflora and several studies have shown that a reduction in gastric acid secretion is associated with bacterial overgrowth in the upper GIT (Howden and Hunt, 1987; Husebye, 2005; Kanno *et al.*, 2009). A recent study showed that a reduction in gastric acid secretion in a specific rat model led to a significant increase in the population of the lactobacilli group (Kanno *et al.*, 2009). Furthermore, in the same study, a similar effect was observed in a group of human subjects with *Helicobacter pylori*- induced chronic atrophic gastritis, the main cause of acquired human hypochlorhydria, "probably reflecting a failure of the mechanism restricting the intestinal colonization of these bacteria". These results highlight the importance of the gastric acid barrier in maintaining normal gut microbial populations.

1.2. The composition of the human GIT microbiota

A great number of studies aimed to understand the role of the microbiome in human health, like *Human Microbiome Project* (HMP) (http://commonfund.nih.gov/hmp/), *Metagenomics of the Human Intestinal Tract* (Meta-HIT) (http://www.metahit.eu/) and *International Human Microbiome Consortium* (IHMC) (http://www.human-microbiome.org/), have shown that human gastrointestinal microbiota is prevalently constituted by bacteria. It has been estimated that the human gut is populated with as many as 100 trillion cells, tenfold the number of human cells (Ley *et al.*, 2006). The human gut microbiota metagenome spans 150 times the genes present in the human genome (Qin *et al.*, 2010). Metagenomic sequencing studies with faecal samples of European individuals showed that over 99.1% of the genes were of bacterial origin (between 1,000 and 1,150 prevalent bacterial species and each individual harboring at least 160 species), the rest of the genes being mostly archaeal, and only 0.1 % were of eukaryotic and viral origins (Qin *et al.*, 2010).

Among bacteria, the human gut microbiota is dominated by species from the phyla *Firmicutes* and *Bacteroidetes* (Table 1) (Mahowald *et al.*, 2009; Marchesi, 2010). The bacterial populations associated with the SI include the phylum *Bacteroidetes*, *Clostridiales* clusters XIV and IV and members of the

Enterobacteriaceae. The populations associated to the LI belong mainly to *Clostridiales* (clusters IX, XIV, and XVI) and *Bacteroidetes (Walter and Ley, 2011).* Other phyla represented in the LI include the *Actinobacteria*, which can be common in the feces of some individuals , the *Verrucomicrobia* (i.e., *Akkermansia*, *Victivallis*), and a number of less abundant phyla such as the *Proteobacteria* and *Fusobacteria* (Walter and Ley, 2011). In the LI, the *Archaea* are represented primarily by *Methanobrevibacter smithii* and, to a lesser degree, by *Methanosphaera stadtmanae*. Other microbes present in the LI include protozoans and fungi, and viruses, mostly prophages and phages whose hosts are prominent bacterial members of the microbiota (Walter and Ley, 2011).

Members of the microbial communities in the GIT can be ecologically classified as autochthonous (indigenous) and allochthonous (nonindigenous) (Table 1) (Savage, 1977). The main difference between autochthonous and allochthonous species is that the former naturally colonizes the habitat, whereas the later cannot colonize it except under abnormal or atypical situations. The autochthonous microbes are always present in the normal adult´s GIT, play a role in maintaining the stable bacterial populations in the GIT, colonize particular parts of the tract, can grow anaerobically, colonize their habitats in succession in infants, and often associate intimately with the gastrointestinal mucosal epithelium. On the other hand, allochthonous species are not characteristic of the normal habitat but are transient microbes which will not be established, but would just be passing through, having arrived in the habitat in food, in water, from another habitat in the GIT, or from elsewhere in the body. These microbes very rarely can establish themselves since they may be outcompeted by autochthonous microbes or killed by host or bacterial factors. However, the allochthonous microbes might colonize the habitats vacated by the autochthonous microbes in a disturbed gastrointestinal system (Ouwehand, 2006).

While the microbial communities present in the LI are quite stable, relatively uniform and well-mixed along the length of the colon (Zoetendal *et al.*, 2002; Eckburg *et al.*, 2005), and to a large degree dominated by autochthonous microbes (Walter and Ley, 2011), the population of the SI is

less stable over time (Hayashi *et al.*, 2005; Booijink *et al.*, 2010). For example, from all the phylotypes detected in the human stomach, only *Helicobacter pylori* persists in this ecosystem, it is absent from the oral cavity or food, and it possesses the phenotypic and genomic traits to survive in the stomach, while other bacterial groups detected in the stomach and SI are allochthonous microbes originating from the oral cavity (Walter and Ley, 2011).

Table 1. Dominant types of microbes either allochthonous or autochthonous in the major habitats of the human gastrointestinal tract. Adapted from *The Human Gut Microbiome: Ecology and Recent Evolutionary Changes* (Walter and Ley, 2011).

Portion of the human GIT	Bacterial population present	
	Autochthonous	Allochthonous
Oral cavity	*Gemella (e.g., G. haemolysans), Granulicatella, Streptococcus (e.g., S. mitis), Veillonella, Prevotella, Porphyromonas, Rothia, Neisseria, Fusobacterium,* ***Lactobacillus***	Allochthonous microbes are generally outnumbered by autochthonous microbes.
Stomach	*Helicobacter pylori*	*Gemella* (e.g., *G. haemolysans*), *Granulicatella*, *Streptococcus* (e.g., *S. mitis*), *Veillonella*, *Prevotella, Porphyromonas, Rothia, Neisseria, Fusobacterium,* ***Lactobacillus***
Small intestine	*Escherichia coli, Klebsiella, Enterococcus, Bacteroides, Ruminococcus, Dorea, Clostridium, Coprococcus, Weissella,* ***Lactobacillus*** (some species)	*Granulicatella, Streptococcus* (ex., *S. mitis*), *Veillonella,* ***Lactobacillus***
Large intestine	Five major phyla: *Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia, and Proteobacteria.* Hundreds of species.	Allochthonous microbes are generally outnumbered by autochthonous microbes.

2. LACTIC ACID BACTERIA

2.1. Definition and classification

Lactic acid bacteria are a heterogeneous group of microorganisms which produce lactic acid as the major sugar fermentation product. After many years of controversy regarding the classification, today, the term lactic acid bacteria is commonly used to refer to two phylogenetically distant bacterial groups: *Lactobacillales* (Firmicutes) and *Bifidobacteriales* (Actinobacteria). A classification of both groups with the more relevant genera concerning feeding and health is shown in Table 2.

2.2. Bifidobacteriales

This group goes beyond the subject of this PhD thesis thus only a brief description will be given here, the reader is referred to reviews on the subject for more information (Poupard *et al.*, 1973; Biavati and Mattarelli, 2006).

The genus *Bifidobacterium* is usually known as bifidobacteria and groups more than 30 species. They are Gram-positive, catalase negative, oxidase negative and anaerobic, however some species can grow in the presence of O_2 if CO_2 is also present. Bifidobacteria are characterized by the use of a special metabolic route for hexose fermentation known as “fructose-6-phosphate shunt” or “bifidus shunt”(Figure 2A), where the key enzyme (fructose-6-phosphate phosphoketolase) of this pathway, splits hexose phosphate to erythrose-4-phosphate and acetyl phosphate (Schramm *et al.*, 1958). This enzymatic activity is a key characteristic that allows differentiating bifidobacteria from the rest of lactic acid bacteria (*Lactobacillales*). The presence of bifidobacteria in the gastrointestinal and genital tracts of human (adults and infants) and animals has stimulated much interest among bacteriologists and nutritionists (Biavati and Mattarelli, 2006).

Table 2: Lactic acid bacteria taxonomy classification according to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/guide/taxonomy/). Adapted from *¿Qué son las bacterias lácticas?* (Aznar and Zúñiga, 2011).

Lactic Acid Bacteria taxonomy classification						
Kingdom	**Filo**	**Class**	**Subclass**	**Order**	**Family**	**Genus**
Bacteria	*Actinobacteria*	*Actinobacteria*	*Actinobacteridae*	*Bifidobacteriales*	*Bifidobacteriaceae*	*Bifidobacterium*
Bacteria	*Firmicutes*	*Bacilli*		*Lactobacillales*	*Aerococcaceae*	
					Carnobacteriaceae	*Carnobacterium*
					Enterococcaceae	*Enterococcus*
					Lactobacillaceae	*Lactobacillus*
						Pediococcus
					Leuconostocaceae	*Leuconostoc*
						Oenococcus
						Weissella
					Streptococcaceae	*Lactococcus*
						Streptococcus

2.3. Lactobacillales

The order Lactobacillales (hereafter referred to as LAB) is constituted by 6 families that comprise 19 genera (Table 2). LAB are Gram-positive rods and cocci microorganisms, non sporulanting Firmicutes (low G+C DNA content (<50%)), that produce lactic acid as a major or sole fermentation product. The members of this group lack porphyrins and cytochromes, do not carry out oxidative phosphorylation, and hence obtain energy mostly by substrate-level phosphorylation. Most LAB grow anaerobically, however many of them tolerate oxygen and can grow in its presence, being called aerotolerant anaerobes. Most of them obtain energy primarily from the metabolism of sugars and have limited biosynthetic abilities (requiring amino acids, vitamins, purines and pyrimidines for growth), thus they are found in nutrient-rich niches.

LAB have been classically divided depending on the pattern of products formed from the fermentation of sugars (Figure 2, B and C): **homofermentative LAB** that mostly produce lactic acid, and **heterofermentative LAB**, that produce a mixture of lactate and other products, mainly acetate, ethanol and CO_2. However, this characteristic is not related to the phylogenetic relationships among LAB. The differences are mainly due to the presence or absence of the aldolase enzyme, one of the key enzymes of glycolysis. Homofermentative LAB contain aldolase and produce two molecules of lactate from glucose by the glycolytic pathway. Heterofermenters lack Fructose-1, 6-bisphosphate aldolase, so glucose follows a different pathway that leads to the pentose phosphate pathway: they oxidize glucose 6-phosphate to 6-phosphogluconate and then decarboxylate this to pentose phosphate, which is converted to triose phosphate and acetyl phosphate by the key enzyme phosphoketolase. In heterofermenters, triose phosphate is converted to lactic acid with the production of ATP and the acetyl phosphate produced is used as an electron acceptor that is reduced by NADH (generated during the production of pentose phosphate) to ethanol. This occurs without ATP synthesis because the energy-rich CoA bond is lost during ethanol formation. Another difference between homo- and hetero-fermenters

is that the later produce CO_2 as a consequence of the decarboxylation of 6-phosphogluconate; homofermenters do not produce CO_2.

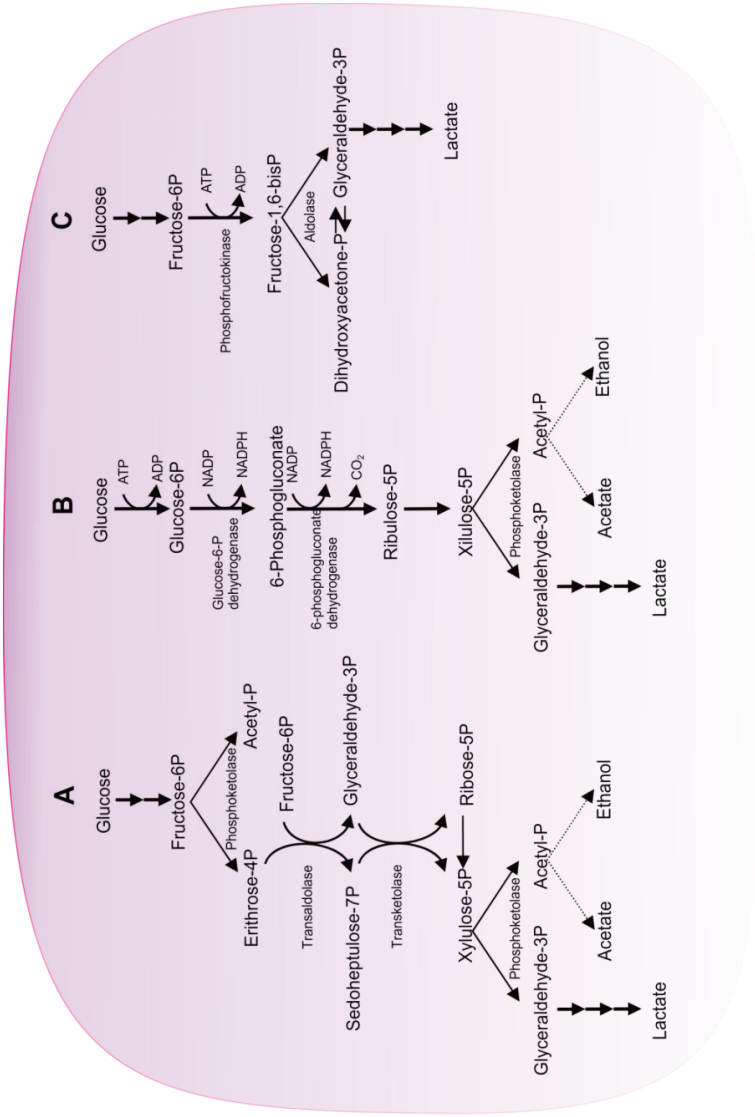


Figure 2. Glycolytic routes of lactic acid bacteria (only the more relevant reactions are showed). A, "bifidus shunt"; B, heterofermentative and C, homofermentative routes of *Lactobacillales*. From *¿Qué son las bacterias lácticas?* (Aznar and Zúñiga, 2011)

2.3.1. The genus *Lactobacillus*

Lactobacilli are typically rod-shaped, varying from long and slender to short, bent rods. Among them, homofermentative, heterofermentative or facultative heterofermentative species can be found. As most LAB, lactobacilli are fastidious organisms with numerous nutritional requirements, therefore they are commonly found in rich-nutrient carbohydrate-containing habitats such as foodstuffs, plant materials and also associated to human and animal mucosas (Hammes and Hertel, 2006). *Lactobacillus* are detected within adult and infant fecal samples, nevertheless the level of lactobacilli are low in the colon and they are more prevalent in the stomach and specially the SI (Kleerebezem and Vaughan, 2009; Walter and Ley, 2011). They are rarely, if ever, pathogenic. Lactobacilli are commonly found in foodstuffs from animal origin, like dairy products and fermented meat products, as well as from vegetal origin, such as fermented vegetables and beverages.

Lactobacillus casei is a facultative heterofermentative lactic acid bacterium commonly found in food (raw and fermented milk, meat products, vegetables and sourdough breads). It produces lactic acid as the major end product of carbohydrate metabolism and it is of industrial interest since it is used as a starter culture in dairy products and for maturation of some types of cheeses. *L. casei* is also found in the oral cavity, gastrointestinal and genital tracts of humans and other animals. Some strains are considered as probiotics (de Vrese and Schrezenmeir, 2008; Kleerebezem and Vaughan, 2009) and are commercialized in food products like Yakult® and Actimel®.

Taxonomic classification of *Lactobacillus casei* has been a matter of debate. Taxonomic studies have shown that the type strain (*L. casei* ATCC393) is phylogenetically distant from the rest of strains classified within the same species, which constitutes a homogeneous taxon. As a consequence, it was proposed to create a new species for these isolates, *Lactobacillus paracasei* (Collins *et al.*, 1989). It was also proposed that *Lactobacillus casei* ATCC 334 and *Lactobacillus paracasei* strains were members of the same taxon and therefore could be united within the name *Lactobacillus casei*, the name *Lactobacillus paracasei* being rejected, and designating ATCC 334 as the neotype strain for the species *Lactobacillus casei* (Dellaglio *et al.*, 2002).

Recently, the Judicial Commission of the International Committee on Systematics of Bacteria, rejected to create a new type strain for *L. casei* and accepted the creation of a new species *L. paracasei* (type strain *L. paracasei* ATCC25302). This situation led to confusion and today the name *L. casei* is used as a synonymous of *L. paracasei* in many works and strains. Several genomes of *L. casei* strains are sequenced or being sequenced at the moment (ex., strains ATCC334, BL23, Zhang, etc.) (Cai *et al.*, 2009; Mazé *et al.*, 2010; Toh *et al.*, 2013).

Lactobacillus casei BL23 is the strain subject of the present study. Its origin is uncertain (Acedo-Félix and Pérez-Martínez, 2003) however its genome has been recently sequenced (3.1 Mb) (Mazé *et al.*, 2010) and it has been used by several laboratories in genetic and physiological studies for many years (Rochat *et al.*, 2007; Muñoz-Provencio *et al.*, 2010; Watterlot *et al.*, 2010; Alcántara and Zúñiga, 2012; Bourand *et al.*, 2013; Landete *et al.*, 2013; Palomino *et al.*, 2013).

2.4. Probiotics

Probiotics are defined as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (Food and Agriculture Organization of the United Nations & World Health Organization, 2006).

Fermentation of milk, vegetables and meat products has been used for thousands of years in many cultures to prevent food spoilage without even knowing the existence of microorganisms. The link between lactobacilli and human health was first proposed by Metchnikoff (Gordon, 2008) who claimed in his book "The prolongation of life: optimistic studies" that the intake of lactobacilli-containing yogurt results in a reduction of toxin-producing bacteria in the gut and this is associated with increased longevity of the host. Tissier recommended the administration of bifidobacteria to infants suffering from diarrhea, claiming that bifidobacteria supersede the putrefactive bacteria causing the disease (Tissier, 1907). He showed that bifidobacteria were predominant in the gut of breast-fed infants. Shirota isolated the *Lactobacillus casei* Shirota strain and started to commercialize it like Yakult. These studies

where the beginning of the recognition of probiotic properties of some strains of LAB. The majority of probiotic microorganisms belong to the genera *Lactobacillus* and *Bifidobacterium* however, some strains belonging to the genera *Streptococcus*, *Enterococcus, Escherichia*, *Bacillus* as well as some *Saccharomyces* yeasts are also considered as probiotics (de Vrese and Schrezenmeir, 2008; Kanmani *et al.*, 2012).

An intensive scientific effort to demonstrate the beneficial effects of probiotics just started in the 80s of XX century, speeding up since year 2000 aimed to use the knowledge on the human microbiome in the early detection of chronic diseases, personalized medicine and more healthful food (de Vrese and Schrezenmeir, 2008; Clarke *et al.*, 2012; Kanmani *et al.*, 2012; Makinen *et al.*, 2012; Nagpal *et al.*, 2012; Robles Alonso and Guarner, 2013; Sanders *et al.*, 2013). The maintenance of the right equilibrium of the microbiota is necessary for the maintenance of the homeostasis of a healthy individual. Gut dysbiosis are produced when an imbalance in the intestinal bacteria exists that allows different subdominant opportunistic bacteria to overgrow leading to a situation of illness. Dysbiosis has been related with obesity and diabetes (Martin *et al.*, 2013). Dysbiosis has also been related (between other factors: genetic components, immunological disorders, environmental factors, pathogens and microbiota) with inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC). IBDs are characterized by an abnormal activation of the immune system associated with the gut, resulting in a chronic inflammation of the digestive system (Martin *et al.*, 2013).

Even without a permanent colonization of the human GIT, lactobacilli can benefit the health of consumers as follows (Reviewed in (Hammes and Hertel, 2006)): 1) Dairy products containing LAB, including lactobacilli, are better tolerated by persons with lactose intolerance, since the bacterial β-galactosidase supports the hydrolysis of lactose in the SI (Fernandes *et al.*, 1992; Saavedra, 2001). 2) Preparations containing lactobacilli have been shown effective in the treatment of diarrhea of various etiologies (Fernandes *et al.*, 1992; Saavedra, 2001). 3) Some lactobacilli can exert effects on the immune system of the host like modulation of cytokine gene expression, stimulation of phagocytosis by peripheral blood leucocytes, and an increase of serum IgA and

IgM titers (Schiffrin *et al.*, 1995; Haller *et al.*, 2000; von Schillde *et al.*, 2012). 4) Some lactobacilli can reduce the levels of pro-carcinogenic enzymes in feces and have the potential to bind and degrade carcinogens (Fernandes *et al.*, 1992; Hirayama and Rafter, 1999). 5) Encouraging results have been obtained with the use of probiotics (including lactobacilli strains) in IBD therapy, for example, lactobacilli were shown to reduce mucosal inflammation and to enhance the mucosal barrier function in an experimental murine model (IL-10 knock-out mice) (Madsen *et al.*, 1999; Schultz *et al.*, 2002), and the probiotic preparation so-called "VSL-3" (consisting of mixture of strains of *L. casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* spp. *bulgaricus*, bifidobacteria and *Streptococcus thermophilus*) was highly effective in maintenance of remission in human patients suffering from chronic pouchitis and UC (Gionchetti *et al.*, 2000; Tursi *et al.*, 2010).

In particular, L. casei BL23 has shown anti-inflammatory effects after oral administration in mice IBD models (Benoit Foligne and Joelle Dewulf, 2007) as well as in a murine DSS-induced colitis model (Rochat *et al.*, 2007). Muñoz-Provencio *et al*. also showed that this strain adheres to human resected intestinal mucosa, a characteristic considered of relevance for a probiotic to exert its beneficial health effects (Muñoz-Provencio *et al.*, 2009). In a different study, *L. casei* BL23 (referred to as *Lactobacillus paracasei* BL23 in this study) together with *L. plantarum* 299v were shown to restore homeostasis and decreased signaling events occurring during T cell activation in an inflammation colon explant *ex-vivo* culture model (Bäuerl *et al.*, 2013).

Administration of probiotic microorganisms requires an exhaustive evaluation of their security thus several regulations exist in this matter like the QPS (*Qualified Presumption of Safety*) from the EFSA (*European Food Safety Authority*) and the GRAS (*Generally Recognized as Safe*) from the FDA (*American Food and Drug Administration*). The ability of probiotic microorganisms to survive to their transit through the gastrointestinal tract (tolerance to gastric and bile acid, and resistance to digestive enzymes) as well as industrial processing of food and storage, in order to arrive to their place of action in sufficient amounts, in a viable and active state, is of major importance and one of the selection criteria for probiotic strains (Food and

Agriculture Organization of the United Nations & World Health Organization, 2006). Due to the interest of lactobacilli as probiotic and industrial bacteria, numerous studies address their efforts to determine the mechanisms of stress response enhancing their survival against the environmental challenges they are exposed to (Morel-Deville *et al.*, 1998; Murga *et al.*, 2000; De Angelis and Gobbetti, 2004; Azcarate-Peril *et al.*, 2005; Piuri *et al.*, 2005; Tymczyszyn *et al.*, 2007; Fernandez *et al.*, 2008; Broadbent *et al.*, 2010; Li *et al.*, 2010; Rivas-Sendra *et al.*, 2011; Alcántara and Zúñiga, 2012; Koponen *et al.*, 2012; Wu *et al.*, 2012).

3. CELL SURFACE OF LACTOBACILLUS

The bacteria cell envelope is a complex multilayered structure that serves to protect these organisms from their unpredictable and often hostile environment, and at the same time allows selective passage of nutrients from the outside and waste products from the inside. In 1884 Christian Gram developed a staining procedure that allowed him to classify almost all bacteria in two groups: one group that retained his stain, the Gram-positive, and another group that did not, Gram-negative. The main difference between both groups lies in their cell envelope: while Gram-negative bacteria possess an inner plasma membrane, a thin peptidoglycan cell wall and an outer membrane, Gram-positive only present a plasma membrane and a thick cell wall of peptidoglycan that helps to withstand the turgor pressure exerted by the cytoplasm on the plasma membrane. In this introduction, only the Gram-positive cell envelope present in *Lactobacillus* will be described in further detail.

The cell surface of lactobacilli determines the ability to adhere and interact with the epithelium and the mucosal layers of the GIT (Vélez *et al.*, 2007; Sengupta *et al.*, 2013), as well as to survive the hostile conditions of the luminal environment and the competing microbiota: lactobacilli modify their surface properties in response to environmental changes to maintain bacterial cell integrity (De Angelis and Gobbetti, 2004; Wu *et al.*, 2012). Different strains

of lactobacilli are known to show great diversity in cell surface architecture with strain-specific characteristics (Sengupta *et al.*, 2013). The cell envelope of lactobacilli (Figure 3), is composed of the plasma membrane with embedded proteins, surrounded by the cell wall (Kleerebezem *et al.*, 2010). The cell wall consists of a thick multilayered sacculus made of peptidoglycan, decorated with teichoic acids (wall teichoic acids (WTA) and/or lipoteichoic acids (LTA)), cell wall polysaccharides, pili and flagela (proteinaceous filaments), and cell surface proteins that are anchored to the cell wall through different mechanisms. Some species of lactobacilli display an additional paracrystalline layer of proteins surrounding the peptidoglycan layer, referred to as the S-layer, but it is not present in *L. casei* BL23.

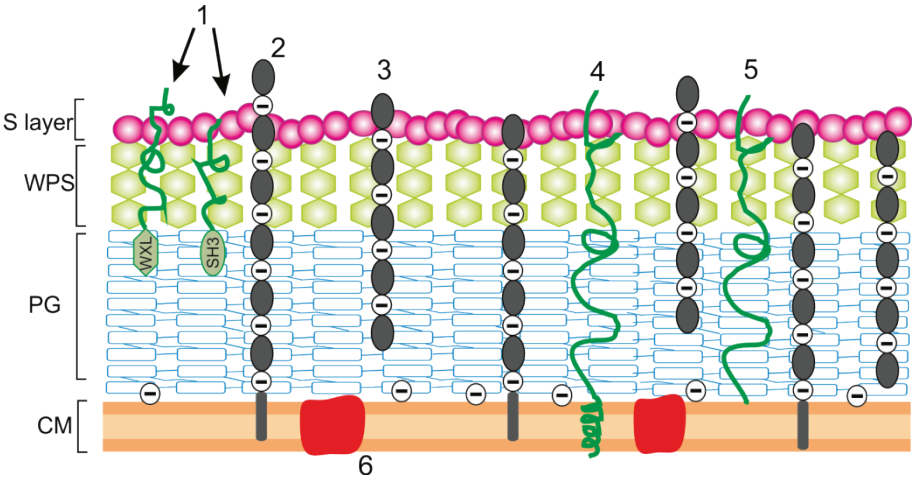


Figure 3. Schematic representation of *Lactobacillus* cell surface. CM, bilipidic cell membrane; PG, multilayered peptidoglycan; WPS, wall polysaccharides; S-layer is also showed. 1, cell wall associated proteins; 2, lipoteichoic acids (LTA); 3, wall teichoic acids (WTA); 4, membrane associated protein (N or C terminally anchored); 5, lipoprotein; 6, cell membrane embedded proteins. Negative net charge of membrane phosphatidylglycerol and LTAs and WTAs is also shown. Adapted from *The biosynthesis and functionality of the cell-wall of lactic acid bacteria* (Delcour *et al.*, 1999) and *The role of cell surface architecture of Lactobacilli in host-microbe interactions in the gastrointestinal tract* (Sengupta *et al.*, 2013).

3.1. The Cell Wall of Lactobacillus

As other Firmicutes, the *Lactobacillus* cell wall consists of a thick multilayered sacculus of peptidoglycan interwoven with teichoic acids and polysaccharides. The cell wall also contains proteins anchored through different mechanisms. The cell wall prevents the osmotic lysis, it determines the bacterial shape and it is a protective layer against toxic substances.

3.1.1. Peptidoglycan

Composition and Structure

Peptidoglycan is the main component of the cell wall. It is composed of glycan strands consisting in their unmodified form of alternating residues of β-1-4-linked N-acetyl muramic acid (NAM) and N-acetylglucosamine (NAG) cross-linked by short peptides. The D-lactoyl residue of the NAM is substituted by a pentapeptide ending in D-Ala-D-Ala or pentadepsipeptide ending in D-Ala-D-Lac (D-Lac, D-lactate), whose composition in lactobacilli in its unmodified form is L-Ala$^{(1)}$-γ-D-Glu$^{(2)}$-(L-Lys or *meso*-A_2pm or L-Orn)$^{(3)}$-D-Ala$^{(4)}$-(D-Ala or D-Lac)$^{(5)}$ (Kleerebezem *et al.*, 2010). Many modifications of this basic composition are found in the glycan strands and its associated stem peptides. The number of peptidoglycan layers is variable being this sacculus between 20 and 80 nm thick. The length of the peptidoglycan chains, the crosslinking degree and the modifications vary depending on the species, strain and growth conditions.

Recent electron cryotomography studies with the model Gram-positive microorganism *Bacillus subtilis* (Beeby *et al.*, 2013) showed that peptidoglycan is a uniformly dense layer with a textured surface. These results support that the glycan strands in Gram-positive cell wall run circumferentially around the cell and are cross-linked by peptides running approximately parallel to the long axis of the cell, just as they do in Gram-negative cells. The authors further conclude that this architecture is assembled inside-to outside, through deposition of glycan strands on the inner face of the sacculus and degradation of the outer surface. Concerning the existence of a Gram positive periplasmic space, *Beeby et al.(Beeby et al., 2013),* compare their recent results with contradicting results of previous studies using high-pressure freezing electron

cryo-microscopy (Matias and Beveridge, 2005) and suggest that the Gram positive periplasm may be dynamic: with a fairly rigid cell wall, the volume of the cell inside may vary in response to buffer conditions and/or pressure, at times opening up a periplasmic space.

Biosynthesis

The biosynthetic cycle of the peptidoglycan polymer that constitutes the bacterial cell wall is initiated on the cytoplasmic side by assembly of precursor molecules and their attachment to the lipid carrier undecaprenyl-phosphate (UP). The resulting complex of N-acetylglucosamine-N-acetylmuramyl-pentapeptide, covalently coupled to the lipid carrier via a pyrophosphate linker is referred to as lipid II (Figure 4) (van Heijenoort, 2007). The undecaprenyl moiety of lipid II is used as lipid carrier in several macromolecular syntheses related to the biosynthesis of cell wall components. The main role of lipid II in peptidoglycan biosynthesis is to convey the pentapeptide disaccharide building blocks synthesized in the cytoplasm to their site of polymerization and crosslinking on the external face of the cytoplasmic membrane (Delcour *et al.*, 1999). After flipping of lipid II to the outer face of the cytoplasmic membrane (Mohammadi *et al.*, 2011), the peptidoglycan subunits are incorporated into the growing cell wall. This step is the target for many antimicrobial peptides (AMPs), e.g. the lantibiotics, which bind to the pyrophosphate moiety of lipid II on the outer face of the membrane (Figure 4) (Bonev *et al.*, 2004; Hsu *et al.*, 2004). After removal of the peptidoglycan precursors, the lipid carrier remains in the pyrophosphate form (UPP), which is dephosphorylated by UPP-phosphatases (Bouhss *et al.*, 2008) and flipped back to the cytoplasmic face of the membrane (Figure 4). This recycling step is inhibited by bacitracin, which tightly binds to the pyrophosphate group and thus prevents the dephosphorylation reaction (Figure 4) (Siewert and Strominger, 1967; Storm and Strominger, 1973; Schneider and Sahl, 2010; Economou *et al.*, 2013).

Penicillin-binding proteins (PBPs) are the enzymes that catalyze the final steps of the polymerization (transglicosilation) and cross-linking (traspeptidation) of peptidoglycan, thus they are major players in the biosynthesis of the bacterial cell wall (Ghuysen, 1991).

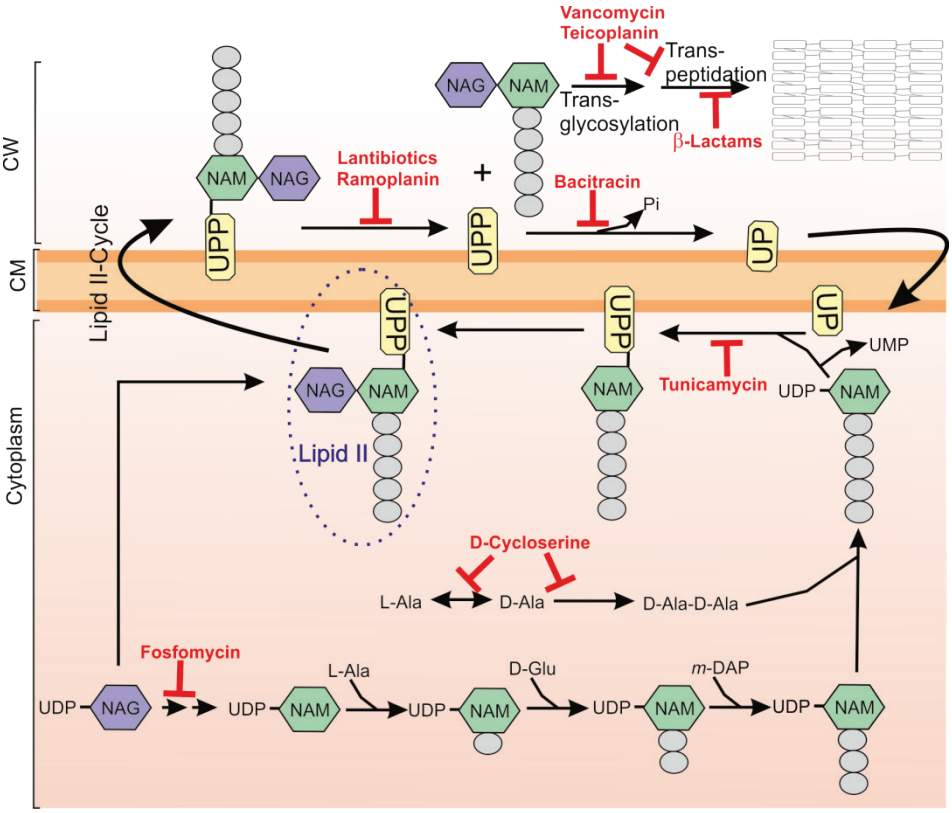


Figure 4. Schematic representation of peptidoglycan biosynthesis and its inhibition by antibiotics. Important steps in cell wall biosynthesis are depicted and their cellular location is indicated on the left. CW, cell wall; CM, cytoplasmic membrane; NAG, N-acetyl-glucosamine; NAM, N-acetyl-muramic acid; UP, undecaprenyl-phosphate; UPP, undecaprenyl-pyrophosphate. Amino acids are symbolized by small grey circles. Lipid II consists of the NAG/NAM-pentapeptide building block, covalently linked to the lipid carrier molecule UP via a pyrophosphate ester bridge. The steps of cell envelope biosynthesis linked to UP are referred to as "Lipid II cycle". Antibiotics are placed next to the step they inhibit. Modified from *Defence against antimicrobial peptides: different strategies in Firmicutes* (Revilla-Guarinos *et al.*, 2014).

Function

The peptidoglycan layer counteracts the turgor pressure of the bacterial cell, avoids large molecules to enter the cell as well as restrict secretion of

large proteins (large proteins depend on the cell wall expansion process to achieve their way out of the cell) (Sengupta *et al.*, 2013). Following biosynthesis, assembly, and incorporation of the peptidoglycan subunits, modifications in the NAG and NAM structures can happen. In lactobacilli, N-deacetylation of NAG/NAM (*L. fermentum*) and 6-O-acetylation of NAM (*L. plantarum, L. casei, L. acidophilus* and *L. fermentum*) of glycan strands has been reported (Kleerebezem *et al.*, 2010). Also, the substitution of C6 of NAM by teichoic and teichuronic acids. These modifications are important for bacterial physiology and microbe-host interactions since they can increase resistance to antibiotics and host-degrading enzymes as well as help to avoid the host innate immunity (Sengupta *et al.*, 2013).

3.1.2. Teichoic acids

Threading through the peptidoglycan layers are long anionic polymers, called teichoic acids (TAs), which are composed largely of glycerol phosphate, glucosyl phosphate, or ribitol phosphate repeats. TAs can be covalently attached to peptidoglycan, the wall teichoic acids (WTAs), or anchored to the head groups of membrane lipids, lipoteichoic acids (LTAs) (Figure 3) (Neuhaus and Baddiley, 2003). In *L. casei* (Neuhaus, 1985), *Lactobacillus rhamnosus* (Perea Vélez *et al.*, 2007) and in *Lactococcus lactis* subsp. *cremoris* (Valyasevi *et al.*, 1990) only LTAs are present.

TAs contribute to the functionality of the cell wall and their functions are varied and species-dependent (Reviewed in (Delcour *et al.*, 1999; Neuhaus and Baddiley, 2003; Swoboda *et al.*, 2010)). The poly (glycerol phosphate) and poly (ribitol phosphate) TAs constitute a reservoir of phosphate and their anionic nature makes them effective scavengers of cations (Mg^{++} in particular). TAs can also help in creating a pH gradient across the cell wall by sequestering the protons expelled through the cytoplasmic membrane as a result of metabolism. TAs influence the activity of autolysins. TAs are immunogens, in a similar way that the lipopolysaccharides of Gram-negative bacteria: a fraction of LTA may be released into the extracellular medium through deacetylation of the lipid anchor, where they are recognized as ligands by receptors present on intestinal epithelial cells.

TAs can account for over 60 % of the Firmicutes cell wall mass and are the major contributors to the anionic character of the Firmicutes surface owing to intercalated phosphate groups (Swoboda *et al.*, 2010). The structure, abundance and the extent of substitution of TAs depends on the species, strain, stage or rate of growth, pH of the medium, carbon source, availability of phosphate, etc. (Delcour *et al.*, 1999). The negative charge density on TAs can be modulated by the addition of D-alanyl substituents by the Dlt system (Neuhaus *et al.*, 1996), which introduces positive charges along the polymer backbone. D-alanine is connected to the alditol residues via an ester bond thus the amino group remains free and positively charged (Figure 10) (Peschel, 2002). D-alanylation is not a general characteristic of TAs, and it is apparently limited to Firmicutes (Neuhaus and Baddiley, 2003).

3.1.2.1. DLT system

The synthesis of D-alanyl-LTA requires four proteins that are encoded by the *dlt* operon (Neuhaus *et al.*, 1996; Neuhaus and Baddiley, 2003). Dcl (encoded by *dltA*) is a D-alanine: Dcp ligase (AMP forming) and Dcp (encoded by *dltC*) is a D-alanyl carrier protein. The other two genes of the operon, *dltB* and *dltD,* encode a transport protein (DltB) and a membrane protein (DltD) that ensures the ligation of D-alanine to Dcp. The mechanism of D-alanylation has not been fully elucidated. One proposed model of D-alanine incorporation is as follows: D-alanine is first activated by the Dcl enzyme in the presence of ATP to yield a D-alanyl adenylate. The same enzyme transfers its D-alanyl moiety to the Dcp carrier protein. The membrane protein DltD provides binding sites for Dcp and Dcl on the cytoplasmic leaflet, and functions in the selection of the correct carrier protein, Dcp, for ligation with D-alanine and in the hydrolysis of mischarged D-alanyl-ACPs. Finally, DltB provides a putative channel for the secretion of D-alanyl-Dcp to the periplasm where D-alanylation occurs. The transfer of the D-alanyl residue from D-alanyl-Dcp to LTA requires only that the acceptor LTA be membrane associated (Neuhaus and Baddiley, 2003). However, other models for the functionality of the Dlt-system have been proposed. The degree of D-alanylation is highly

variable and depends on strain background and growth conditions (Perego *et al.*, 1995; Neuhaus and Baddiley, 2003; McCormick *et al.*, 2011).

Expression of the *dlt* operon is regulated by different mechanisms in different species, and usually it is subjected to the control of several regulatory systems within the same organism. In *Bacillus subtilis*, *dlt* is part of the regulons of the extracytoplasmic-function sigma factors σ^X (Cao and Helmann, 2004; Kingston *et al.*, 2013), σ^V (Guariglia-Oropeza and Helmann, 2011) and the two-component system (TCS) YxdJK (Joseph *et al.*, 2004). In staphylococci, the *dlt* operon is positively regulated by the homologous TCSs GraRS (*Staphylococcus aureus*; (Li *et al.*, 2007)) or ApsRS (*Staphylococcus epidermidis*; (Li *et al.*, 2007) in response to cationic AMPs (CAMPs), and it is repressed by the TCS ArlRS in response to high extracellular concentrations of Mg^{2+}, Ca^{2+} or Na^+ (Koprivnjak *et al.*, 2006). Furthermore, there is evidence indicating that the global regulators Agr (Dunman *et al.*, 2001) and Rot (Saïd-Salim *et al.*, 2003) are also involved in *dlt* regulation in *S. aureus*.

Alterations in the D-alanylation process usually has a dramatic incidence in the functionality of the cell wall as reflected in the diversity of *dlt* mutant phenotypes described in the literature. The *dltB* mutation in *L. plantarum* resulted in increased cell length, damaged dividing cells and perforations of the envelope in the septal region (Palumbo *et al.*, 2006). The inactivation of *dltD* in *L. rhamnosus* GG (ATCC 53103) resulted in 2.4-fold-increased cell length, a low survival capacity in response to gastric juice challenge, an increased sensitivity to human beta-defensin-2, an increased rate of autolysis, an increased capacity to initiate growth in the presence of an anionic detergent, and a decreased capacity to initiate growth in the presence of CAMPs compared to the wild-type (Perea Vélez *et al.*, 2007). *L. casei* mutants defective in LTA D-alanyl esters show aberrant C-shaped morphologies and

defects in cell separation indicative of reduced autolytic activity (Ntamere *et al.*, 1987; Neuhaus *et al.*, 1996). On the contrary, *dltA* mutation in *Lactobacillus reuteri* 100-23 did not have an effect on cell morphology and growth characteristics (Walter *et al.*, 2007).

3.1.3. Cell Wall Polysaccharides

Polysaccharides are usually neutral but they can present an acid character if they are branched with anionic groups like glycerol phosphate. Three groups can be distinguished (Kleerebezem *et al.*, 2010): *(i)* capsular polysaccharides form a thick outer capsule closely associated with the cell wall, *(ii)* wall polysaccharides are covalently attached to NAM of peptidoglycan strands and *(iii)* the extracellular or secreted polysaccharides (Kleerebezem *et al.*, 2010). Distinction between these classes is often difficult and can be dependent on the growth conditions. In *Lactobacillus* they are often heteropolysaccharides (consisting of different sugar moieties such as glucose, galactose, rhamnose, NAG, and N-acetylgalactosamine) however, some strains of lactobacilli are capable of synthesizing homopolysaccharides such as glucans or fructans from sucrose (Kleerebezem *et al.*, 2010). Some polysaccharide chains can serve as anchors for S-layer proteins although their general role is to mediate interactions of lactobacilli with environmental components and promote bacterial adhesion and biofilm formation to inert or living surfaces (Kleerebezem *et al.*, 2010).

3.1.4. Cell Surface Proteins

Cell surface proteins are a diverse group of molecules with important physiological functions in transport and secretion, sensory processes and signal transduction, antibiotic resistance, interaction with the environment, etc. They can be covalently or not-covalently anchored to the membrane or to the cell wall (Figure 3) (Reviewed in detail in (Kleerebezem *et al.*, 2010)). Proteins can be anchored to the membrane by means of N- or C-terminally hydrophobic transmembrane segments, or they can be covalently linked to membrane lipids (lipoproteins) (Figure 3). To the cell wall, they can be covalently anchored to the peptidoglycan though transpeptidation mediated by sortases or they can

be non-covalently anchored by means of binding domains (like LysM (lysine motif), WXL, SH3b…) that help to keep them anchored to different components of the bacterial cell wall (Figure 3).

Many species of lactobacilli display a *surface layer (S-layer)*, made of a crystalline, two-dimensional array of protein or glycoprotein subunits assembled in lattices with different symmetries (Figure 3)(Deepika and Charalampopoulos, 2010). So far, S-layer proteins have not been identified in *L. casei* (Deepika and Charalampopoulos, 2010). Lactobacilli S-layer proteins are highly basic and can represent up to 10-15% of total cell wall proteins. S-layer proteins are noncovalently bound to the underlying peptidoglycan cell wall, generally through secondary polymers such as LTA, WTA, and neutral polysaccharides. S-layer functions are strain specific but some have been described to be involved in adhesion, aggregation and pathogen inhibition (Deepika and Charalampopoulos, 2010).

3.1.5. Pili and Flagella

Pili are nonflagellar appendages resulting from an assembly of multiple pilin subunits. Pili have been functionally analysed and characterized only in *L. rhamnosus* GG (Kankainen *et al.*, 2009). They play a role in bacterial adhesion, invasion, aggregation, formation of biofilms, and modulation of immunity. Flagella (polimers of protein subunits of flagellin) are unusually found in lactobacilli but to date at least twelve motile species of lactobacilli, not the case of *L. casei*, have been recognized (Neville *et al.*, 2012). It is suggested that flagella act as a ligand and mediate activation of signalling pathways and modulation of host immune cells (Sengupta *et al.*, 2013).

3.2. The Cell Membrane

The cytoplasmic membrane is a thin barrier that surrounds the cell and separates the cytoplasm from the cell's environment. If the membrane is

broken the cell dies. It confers little protection from osmotic lysis but it is very effective as a selective permeability barrier being the "gatekeeper" for substances that enter and exit the cell (Madigan *et al.*, 2012). Although some small hydrophobic molecules pass the cytoplasmic membrane by diffusion, polar and charged molecules do not diffuse but instead must be transported. Nevertheless, although water is weakly polar it is small enough to pass between phospholipid molecules in the lipid bilayer, thus it can traverse the membrane in both directions. Protons (H^+) and hydroxyl ions (OH^-) cannot diffuse across the membrane. This characteristic allows establishing a charge separation in which H^+ are separated from OH^- across its surface, giving as a result the proton motive force that drives many energy requiring functions in the cell, including some forms of transport, motility, and biosynthesis of ATP (Madigan *et al.*, 2012).

The general structure of the cytoplasmic membrane is a phospholipid bilayer of 6–8 nanometers wide, somewhat fluid, having a consistency approximating that of low-viscosity oil (Madigan *et al.*, 2012). The phospholipids present in *Lactobacillus* are mainly constituted by phosphatidylglycerol with smaller amounts of phosphatidic acid, diphosphatidylglycerol (cardiolipin), lysylphosphatidylglycerol, phosphoglycolipids and diglycosyldiacylglycerol. The major cell membrane fatty acids of *Lactobacillus* spp. are myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1n(9)}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1n(9)}$), *cis*-vaccinic acid ($C_{18:1n(11)}$), and the cyclopropanes dihydrosterculic acid [*cis*-9,10-methyleneoctadecanoic acid; $C_{19:0(9c)}$] and lactobacillic acid [*cis*-11,12-methyleneoctadecanoic acid; $C_{19:0(11c)}$] (Rizzo *et al.*, 1987). Integral membrane proteins are firmly embedded in the membrane (Figure 3). Other proteins have one portion anchored in the membrane and extra membrane regions that point into or out of the cell. Finally, peripheral membrane proteins, like lipoproteins, are not membrane-embedded but nevertheless remain firmly associated with membrane surfaces by means of a lipid tail that anchors the protein into the membrane. Proteins in the cytoplasmic membrane are usually arranged in clusters to favor the proximity of proteins that need to interact one to another. The overall protein content of the membrane is quite high, and it is thought that the variation in lipid bilayer thickness (6–8 nm) is necessary to

accommodate thicker and thinner patches of membrane proteins (Madigan *et al.*, 2012).

The composition of the membrane varies depending on the environmental conditions. This process is tightly controlled by genetic regulation of the expression of biosynthetic enzymes and biochemical regulation of their activities. Modification of the cell membrane composition involves both the synthesis de novo of FAs and the modification of pre-existing phospholipids (Zhang and Rock, 2008). This regulation includes (Zhang and Rock, 2008): *i)* the control of the total membrane lipid content; *ii)* changes in the proportion of phospholipid polar head groups (balancing the zwitterionic, neutral and acidic phospholipids) when necessary, since phospholipid composition determines membrane-protein topology; *iii)* regulation of the biophysical properties of the membrane bilayer by changing the structure of the membrane fatty acyl chains (saturated : unsaturated FAs ratio, type of branched chain FAs, formation of cyclopropane FAs (CFAs), FAs cis-trans isomerization); and *iv)* the modification of the membrane bilayer charge, for example by the MprF protein.

3.2.1. MprF protein: the multiple peptide resistance factor (MprF) (Peschel *et al.*, 2001) is a large integral membrane enzyme that modifies anionic phospholipids with L-lysine (sometimes also L-alanine) thereby introducing positive charges into the membrane surface. MprF is present in both Gram-positive and Gram-negative bacteria. In *S. aureus*, MprF is composed of 14 transmembrane domains (TMDs) and a cytosolic C-terminal domain. The synthesis reaction happens between a charged lysyl-tRNA and a phosphatidylglycerol (PGly) to form lysyl-phosphatidylglycerol (Lys-PGly). Later the “flipping” of Lys-PGly from the inner to the outer surface of the membrane takes place. Synthesis and translocation functions are allocated in the C- and N- terminal domains of MprF, respectively, and can be separated into two functional proteins (Ernst *et al.*, 2009). *S. aureus* MprF mutants are hardly impaired in growth and show little phenotypic changes under standard laboratory conditions,

nevertheless, they are strongly susceptible to CAMPs such as host defence peptides and bacteriocins (Ernst and Peschel, 2011).

Some of the proteins associated with the membrane are transport proteins that accumulate solutes inside the cell against the concentration gradient or that contribute to export solutes outside of the cell from the cytosol. An important characteristic of transport systems is that their biosynthesis is highly regulated by the cell depending on the requirements at every moment according to the substrate availability. Between all the membrane-associated transport proteins, ATP -binding cassette transporters play an important role in this PhD thesis, thus, they will be described in more detail.

3.2.2. ATP-binding cassette (ABC) transporters are multidomain integral membrane proteins that use the energy of ATP hydrolysis to translocate solutes across cellular membranes in all phyla. They comprise a conserved core structure of two TMDs that form the translocation pore and two cytosolic nucleotide-binding domains (NBDs) that bind and hydrolyze ATP, thereby generating conformational changes that are coupled to the TMDs and ultimately lead to substrate translocation. Nonetheless, diversity in this domain architecture can be found. For extensive reviews on ABC transporters the reader is referred to (Chang, 2003; Jones and George, 2004; Locher, 2004; Hollenstein *et al.*, 2007; Eitinger *et al.*, 2011).

The primary sequences of the NBDs (the molecular motors that transform the chemical potential energy of ATP into protein conformational changes) are highly conserved. In contrast, the TMDs sequences are markedly variable what is thought to be related with the substrate binding specificity. ABC transporters transport a wide variety of molecules (ions, sugars, peptides…) being for that reason involved in diverse cellular processes such as maintenance of osmotic homeostasis, nutrient uptake, etc.

(Jones and George, 2004; Eitinger *et al.*, 2011). Peptide efflux ABC transporters are specific subfamilies of ABC transporters mainly involved in exporting antimicrobial peptides (lantibiotics, bacteriocins and competence peptides)(Havarstein *et al.*, 1995). Most of the bacteriocin encoding genes appear associated with genes encoding for ABC exporters (de Jong *et al.*, 2006).

ABC transporters endowed of regulatory functions have also been described. Like ABC transporters involved in regulating gene expression through sequestration of transcriptional regulators at the membrane (Richet *et al.*, 2012), as well as peptide-7 ABC transporters from Firmicutes involved in mediating signaling (Figure 12) (Dintner *et al.*, 2011; Hiron *et al.*, 2011). The later will be described in detail in section *6.1 Bce-like two component systems*.

4. METHODS FOR ASSESSING BACTERIAL CELL SURFACE CHARGE PROPERTIES.

As previously described, bacterial cell surfaces possess net negative electrostatic charge (Figure 3) due to the preponderance of acidic groups such as carboxylate and phosphate over basic amino groups in the cell envelope components, which can be ionized depending mainly on the environmental pH, conferring electrostatic charge to the cell surface. Knowledge of the physiochemical properties of the cell surface can be helpful in understanding bacterial physiology. However, the electrostatic charge of small particles, such as bacterial cells, cannot be ascertained directly so indirect means are required.

4.1. Binding assays with charged molecules is an easy and relatively quick method to compare the cell surface charge of different bacteria. In this method bacteria are incubated with a negatively charged (acidic) molecule (ex. green fluorescent protein (GFP), calculated pI 5.8 (Peschel *et al.*, 1999)) or

positively charged (basic) molecule (ex. cytochrome *c* (Cyt *c*), calculated pI 10.0 (Peschel *et al.*, 1999; Meehl *et al.*, 2007; Matsuo *et al.*, 2011)). Subsequently the bacteria are removed by centrifugation and the amount of these substances remaining in the supernatants is determined fluorometrically (GFP) or photometrically (Cyt *c*), for example. Qualitative results are obtained by comparison of binding ratios between different strains (like a mutant defective in a cell surface function and the wild type).

4.2. Electrophoretic mobility determinations have also been used to characterize bacterial cell surfaces with regard to the cell overall electrostatic properties (Millsap *et al.*, 1997; Boonaert and Rouxhet, 2000; Hong and Brown, 2008; Ouhara *et al.*, 2008; Deepika *et al.*, 2009; Kłodzińska *et al.*, 2010). Some biophysical considerations need to be explained in order to better understand the principles in which this methodology is based.

The double layer model is used to visualize the ionic environment in the vicinity of a charged colloid (bacteria, in our case) and explains how electrical repulsive forces occur (Figure 5). In Physics and Chemistry, a colloid is a system composed of two or more phases, mainly a continuum fluid phase where a second solid phase (in the form of particles) is dispersed. Negatively charged colloidal particles in suspension attract dissolved cations that form a firmly attached layer around the surface of the particle; this layer of counter-ions is called the *Stern layer (Figure 5).* Additional cations are still attracted by the negative surface, while at the same time they are repelled by the Stern layer and other cations that are trying to approach to the bacteria. This dynamic equilibrium results in the formation of a *diffuse layer* of counter-ions *(Figure 5)*. The counter-ions concentration gradually decreases with distance to the bacteria, until it reaches equilibrium with the counter-ion concentration in the solution. On the contrary, negative ions (*co-ions*) concentration gradually increases with distance, until equilibrium is reached. The diffuse layer can be visualized as a charged atmosphere surrounding the particle. The attached counter-ions in the Stern layer and the charged atmosphere in the diffuse layer constitute the *double layer (Figure 5)*.

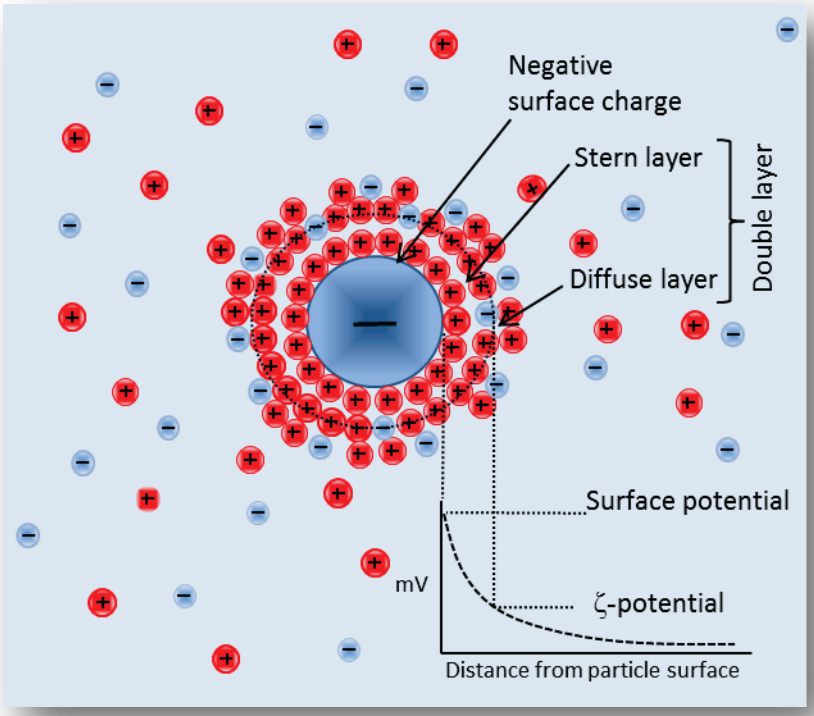


Figure 5. Schematic representation of the Double Layer. The diagram shows the ionic concentration and potential difference as a function of distance from the charged surface of a particle suspended in a dispersion medium. The distribution of positive and negative ions around the charged colloid is shown. Adapted from Wikipedia (http://en.wikipedia.org/wiki/Zeta_potential) and from *Zeta Potential: A Complete Course in 5 Minutes* (http://www.zeta-meter.com/5min.pdf).

The double layer is formed in order to neutralize the charged surface of the particle and, in turn, it causes an electro kinetic potential between the surface of the particle and any point in the mass of the suspending liquid. This voltage difference is the surface potential which is related to the surface charge and the thickness of the double layer. The surface potential approaches zero at the imaginary boundary of the double layer.

Charged dispersed particles will move with a fixed velocity relative to the surrounding fluid in a spatially uniform electric field; this phenomenon is known as *electrophoresis*. The particle´s mobility (direction and rate) is dependent on properties of the suspending liquid (ionic strength, temperature, pH) as well as on the electric field intensity and the electrical potential at the boundary between the moving particle and the liquid. This boundary is called *the slipping plane* or *the shear plane*: the point where the Stern layer (rigidly attached to the bacteria –colloid–) and the diffuse layer (not rigidly attached) meet. In other words, the slipping plane is the distance from the surface to the distance in solution where the solvent molecules are not bound to the surface and are not moving as a unit with the particle. The electrical potential at this junction (plane of shear) is related to the mobility of the particle and is called the *zeta potential (ζ-potential) (Adamson, 1982)* . The *ζ-potential* is somewhere close to the shear plane, at the boundary of the diffuse layer, and it represents the ‘‘effective location’’ of the solid–liquid interface (Figure 5) (Hunter, 1993). Net cell surface charge can be assessed on the basis of *ζ-potential* which can be estimated by measuring cellular electrophoretic mobility in an electric field (Mozes, 1990).

Diverse methods have been used to estimate *ζ-potential* (reviewed in (Wilson *et al.*, 2001)). Microelectrophoresis is one of the first methods employed. It consists in the placement of a cell suspension in an electrophoresis cell, application of voltage across the cell, and direct microscopic observation of the movement of individual bacteria over a given distance, the velocity of which is used to calculate electrophoretic mobility (Moyer, 1936; Brinton, 1959). Electrophoretic mobility is subsequently used to calculate *ζ-potential* values and estimate cell surface charge. The main disadvantage of this method is that tracking individual cells over time is laborious and time consuming.

Other methods have been used for determining charge properties: electrostatic interaction chromatography, aqueous two-phase partitioning, isoelectric equilibrium analysis and electrophoretic light scattering (Wilson *et al.*, 2001). The electrophoretic light scattering (ELS) has proven to be a valuable, rapid and easy method for estimating *ζ-potential* for the

characterization of bacterial cell surface charge properties. In ELS the velocity of particles moving in an electric field is directly measured by determining the frequency change of the laser light they scatter, thereby yielding their electrophoretic mobility (Blake *et al.*, 1994).

5. STRESS RESPONSE

A broad and operational definition of stress was given by Hazen *et al*. (Hazen and Stahl, 2006): *any deviation from optimal growth condition that results in a reduced growth rate or lower level of biomass.* This criterion has been applied throughout the development of this PhD thesis.

Lactobacilli used in food production and as probiotics face a great variety of physicochemical stresses acting over the cell surface. First, during the industrial processing and food storage (ex., osmotic stress, temperature changes, acidification during fermentation of foods and beverages); later, during their passage through the gastrointestinal tract (ex., acid stress in the stomach, bile and pancreatic proteolitic enzymes in the small intestine, mucus, along with starvation stress). Usually the stress response involves gene regulation to improve stress tolerance by altering cellular processes such as cell division, membrane composition, transport systems, metabolism, and DNA metabolism. The specific response varies depending on the strain, species and type of stress.

While different studies have addressed the stress response of lactic acid bacteria (van de Guchte *et al.*, 2002; Spano and Massa, 2006; Bron and Kleerebezem, 2011; Wu *et al.*, 2013) , only a few studies have focused on *Lactobacillus casei* (Neuhaus, 1985; De Angelis and Gobbetti, 2004; Piuri *et al.*, 2005; Broadbent *et al.*, 2010; Li *et al.*, 2010; Rivas-Sendra *et al.*, 2011; Wu *et al.*, 2012; Palomino *et al.*, 2013). Furthermore, whereas the mechanisms involved in the cell envelope stress response (CESR) have been extensively studied in model organisms like *E. coli* and *B. subtilis*, where TCS and extracytoplasmic function (ECF) σ factors are the main players (Heimann, 2002;

Ruiz and Silhavy, 2005; Rowley *et al.*, 2006; Jordan *et al.*, 2008), the mechanisms regulating the stress response in lactic acid bacteria are little known and in *Lactobacillus* they have not been characterized so far.

Different mechanisms of resistance to a variety of physicochemical stressors have been reviewed in detail (van de Guchte *et al.*, 2002; De Angelis and Gobbetti, 2004; Corcoran *et al.*, 2008). For reasons of conciseness and relatedness to the content of this PhD thesis only mechanisms involved in acid stress response and antimicrobial peptide stress response will be described in detail in this section.

5.1. Low pH response.

Growth of lactic acid bacteria is determined, among other factors, by the maintenance of the pH of the media about the value that a specific strain can tolerate (Hutkins, 1993). In particular, growth of most lactic acid bacteria is slower under acidic conditions, and they can suffer acid damage and loss of cell viability (Hutkins, 1993; Walter and Ley, 2011). From the technological point of view this last characteristic can be very useful since inhibition of lactic acid bacteria growth by acidic pH can help to prevent over-acidification and thus flavor defects of the dairy products, once the desired final pH is reached. Nevertheless, when cultures are added to food products with a therapeutic purpose, as is the case of probiotic strains, acid resistance mechanisms that allow the bacteria to survive and maintain cell viability during their storage in the fermented dairy products and during their transit through the acid stomach environment, are desirable and necessary (Hutkins, 1993; Corcoran *et al.*, 2008). When considering their use as probiotics, lactobacilli and bifidobacteria are assayed for resistance to acid at pH values ranging from 2 to 3 (Corcoran *et al.*, 2008).

Lactobacillus strains are usually exposed to different kinds of acid stress: weak organic acids (such as lactic acid and acetic acid, used as preservatives in foods) whose undissociated forms can traverse the membrane lipid bilayer and dissociate in the cytoplasm lowering the intracellular pH, and nonpermeant inorganic acids (as HCl) (Cotter and Hill 2003).

Lactic acid (pKa 3.86), the major end product of fermentation in *Lactobacillus*, is exported out of the cell as lactate ion. As cells proliferate, the pH of the media (pH_{out}) progressively decreases, and as a consequence, the concentration of the undissociated form of lactic acid that can enter the cytoplasm by simple diffusion increases (Lambert and Stratford, 1999). At the more alkaline cytoplasmic pH lactic acid dissociates again leading to an acidification of the cytoplasm (Lambert and Stratford, 1999)). As other bacteria, *Lactobacillus* maintain a cytoplasmic pH (pH_{in}) more alkaline than the external pH (pH_{out}) (Kashket, 1987), thus a pH gradient is generated (ΔpH). But in contrast to other bacteria, *Lactobacillus* does not maintain a constant pH_{in}, instead, they maintain a constant ΔpH (Kashket, 1987). When a critical pH_{in} is reached, the ΔpH cannot be maintained and cellular functions are impaired: growth stops and this determines the entry in stationary phase, even if nutrients are still available (Kashket, 1987; Hutkins, 1993). Kashket (1987) reported that pH_{in} in *L. casei* decreases as pH_{out} decreases, but a ΔpH of around 1 pH unit is maintained and cells grow until pH_{in} reaches values around 4.4 being the pH_{out} around 3.5 (Kashket, 1987). It has also been described that during a change in pH_{out} the pH_{in} of the acid tolerant *Lactobacillus delbrueckii* subsp. *bulgaricus* decreases faster than in the moderately acid-tolerant *Lactococcus lactis* subsp. *lactis*, so it was suggested that lactobacilli may not actively regulate pH_{in} until the pH_{out} is low (Siegumfeldt *et al.*, 2000).

Several mechanisms contribute to regulate the homeostasis of pH_{in} in lactic acid bacteria and the response to acid stress (reviewed in (Hutkins, 1993; van de Guchte *et al.*, 2002; Cotter and Hill, 2003; De Angelis and Gobbetti, 2004; Corcoran *et al.*, 2008)). The mechanisms induced in response to acid shock and acid adaptation can be mediated by TCS (Morel-Deville *et al.*, 1998; Azcarate-Peril *et al.*, 2005).

A broad acid stress response is ***the adaptive acid tolerance response (ATR),*** *which* protects bacteria from acid killing. A brief exposure of the cells to a sublethal extracellular pH (acid adaptation) induces physiological changes that allow the bacteria to survive to a subsequent exposure to a lethal extracellular pH (acid challenge) (Goodson and Rowbury, 1989). Some of the physiological changes occurring during the ATR are described below. The ATR

is also induced at the onset of stationary phase. This response is called growth-phase dependent ATR and it is the result of the accumulation of acidic fermentative end products of the bacterial metabolism (Buchanan and Edelson, 1996). The ATR has been described in some species of lactic acid bacteria, including *L. casei* (Hamilton and Svensäter, 1998; Broadbent *et al.*, 2010; Waddington *et al.*, 2010). Two-dimensional gel electrophoresis has generated a wealth of knowledge about the mechanisms behind the ATR of lactic acid bacteria (Corcoran *et al.*, 2008). Between the proteins upregulated in response to acid stress in different microorganisms are chaperones as well as proteins involved in oxidative stress, ATPase subunits, ABC transporters and glycolytic proteins. Heat shock proteins have also been identified. DNA microarray studies have also been used to study the ATR of lactic acid bacteria by examining changes in gene expression during acid stress (Corcoran *et al.*, 2008).

Physiological changes induced under acid stress (Figure 6):

5.1.1. *Changes in cell surface properties.* The permeability to protons of the bacterial membrane is of major importance in acid resistance. When confronted with acid stress, bacteria can respond by changing the FA composition of the membrane, in order to decrease the proton permeability (Figure 6, step 5) (Cotter and Hill, 2003; Corcoran *et al.*, 2008; Broadbent *et al.*, 2010; Wu *et al.*, 2012). For example, an increase in the concentration of saturated FAs and CFA is associated with a decrease in membrane fluidity (Chang and Cronan, 1999; Fozo *et al.*, 2004). Resistance to acid shock in *E.coli* was dependent of the membrane CFA content (Chang and Cronan, 1999).

Besides changes in the membrane, changes in the cell wall can also occur during the acid stress response, like variation in the levels of D-alanylation of TAs (Figure 6, step 4) (previously described; see section *3.1.2.1*). The implication of the Dlt-system in acid response seems to be acid- and organism- dependent. In *S. aureus*, the *dlt*-operon was down-regulated in cells exposed to lactic acid for 3 hours, and up-regulated in cells exposed to HCl stress for 10 minutes (Rode *et al.*, 2010). The authors suggested that the positive charges introduced by the D-alanylation of teichoic acids, exerted a

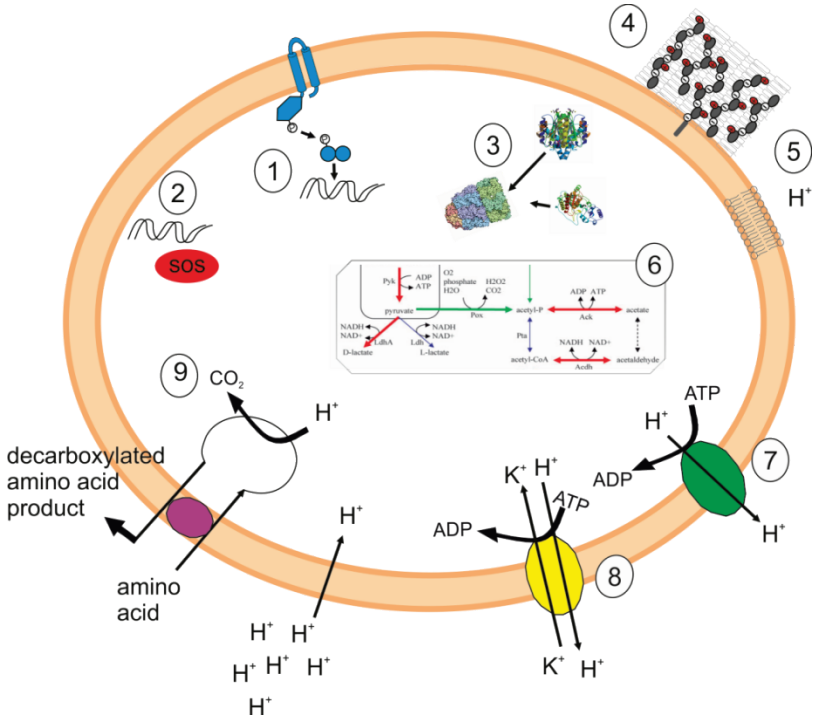


Figure 6. Overview of some of the mechanisms identified in enhancing acid tolerance of bacteria. Acid stress response can be mediated by TCS (1). Induction of DNA and protein repair mechanisms (2 and 3, respectively); changes in cell surface properties (4, D-alanylation of TAs in the cell wall, and 5, changes in cell membrane properties); alteration of metabolic pathways (6); proton pumps (7) and cation transport ATPases (8); production of basic compounds (9). Adapted from *Life Under Stress: The Probiotic Stress Response and How it may be Manipulated* (Corcoran *et al.*, 2008).

physical barrier for H^+ ions by electrostatic repulsion in HCl exposed cells. Broadbent and colleagues observed that cells of *L. casei* ATCC334 down-regulated genes LSEI_0796 (D-Alanyl carrier protein) and LSEI_0797 (D-Alanyl transfer protein DltD precursor) after 20 minutes of acid adaptation at pH 4.5 (Broadbent *et al.*, 2010). In contrast, *Bacillus subtilis* progressively increased the D-alanyl ester content of WTAs in media of decreasing pH from 7 to 5 (Ellwood and Tempest, 1972). It has also been reported that defects in D-alanylation of teichoic acids in *Streptococcus mutants* resulted in acid

sensitivity (Boyd *et al.*, 2000), and a *dltD* mutant of *Lactobacillus rhamnosus* GG also showed diminished acid tolerance compared to the wild type (Perea Vélez *et al.*, 2007). Boyd *et al*. reported that the *dltC* mutation in *S. mutants* resulted in cells more permeable to the passive inflow of protons than wild-type cells (Boyd *et al.*, 2000). Furthermore, the mutant was unable to initiate growth below pH 6.5 compared to pH 5.0 for the wild-type; a higher killing pH (3.5) than the wild-type (3.0) was determined for the mutant strain, and the final pH of *dltC* mutant cultures was slightly higher (4.64) that for the wild-type (4.50) (Boyd *et al.*, 2000). On the contrary, a *dltA* mutant of *Lactobacillus reuteri* 100-23 showed similar growth characteristics as the wild-type, reaching similar final optical density at stationary phase and decreasing the pH of the culture media to the same final pH (4.5), furthermore, the mutant showed the same survival rate than the wild-type when exponentially growing cells were submitted to a sudden acid shift (pH 3); however, the mutant was not able to initiate growth below pH 4, while the wild-type could still grow at 3.5 (Walter *et al.*, 2007). The implication of the D-alanylation of TAs in the acid stress response of *L. casei* BL23 will be addressed in Chapter 3 of this PhD thesis.

5.1.2. *DNA and protein repair mechanisms.* Nucleic acid damage can occur at low pH due to protonation of bases and consequent disruption of glycosidic bonds between pentoses and nitrogen bases. *Molecules involved in DNA* repair are essential for acid resistance in bacteria, such as RecA, that participates in the homologous recombination and regulates the SOS response (Figure 6, step 2) (Cotter and Hill, 2003; De Angelis and Gobbetti, 2004) . Similarly several chaperonins involved in protein turnover are also essential to counteract protein damage upon acid exposure (Figure 6, step 3). Studies of acid adaptation in *Lactobacillus bulgaricus* showed induction of the chaperones GroES, GroEL, HrcA, GrpE, DnaK, DnaJ, ClpE, ClpP, and ClpL, and the repression of ClpC (Fernandez *et al.*, 2008).

5.1.3. *Proton pumps* are also major mechanisms of acid resistance (Figure 6, step 7). F_0F_1-ATPase is a multi-subunit enzyme, comprising a catalytic portion (F_1) for ATP hydrolysis and an integral membrane portion (F_0) that functions as a membranous channel for proton translocation. F_1 incorporates the α, β, γ, δ and ε subunits, and F_0 the a, b, and c subunits. The F_0F_1-ATPase of fermentative

bacteria generates a proton motive force (PMF) via proton extrusion out of the cell, using ATP since protons are expulsed against a concentration gradient (Hutkins, 1993). Physiologically, for every proton pumped out, one ATP is consumed (Lambert and Stratford, 1999). F_0F_1- ATPase is crucial for maintaining pH homeostasis at low pH in lactic acid bacteria. Indeed, F_0F_1-ATPase of lactobacilli appears to function at lower pH values than other lactic acid bacteria (Hutkins, 1993).

Cation transport ATPases such as K^+ ATPase, that exchanges K^+ for intracellular H^+, can also contribute to pH homeostasis (Figure 6, step 8).

5.1.4. Another mechanism to enhance survival in acidic environments is the *production of basic compounds* that leads to an increase in the alkalinity of the cytoplasm (Figure 6, step 9) (van de Guchte *et al.*, 2002; Cotter and Hill, 2003; Corcoran *et al.*, 2008). Amino acid decarboxylation reactions result in the intracellular consumption of one proton while a more alkaline product is transported out of the cell. For example, ornithine decarboxylase catalyses the conversion of ornithine to putrescine and is present in *Lactobacillus* sp. strain 30A. Histidine decarboxylase can promote growth of lactobacilli at low pH. The arginine deiminase pathway (ADI (Cunin *et al.*, 1986)) catalyzes the conversion of arginine into ornithine, ammonia (two per arginine molecule) and CO_2 with the concomitant formation of one mol of ATP per mol of arginine consumed. The NH_3 formed reacts with H^+ to assist in alkalizing the extracellular environment. Urease is also used by a number of bacteria to enhance survival in acidic environments.

Malolactic fermentation (MLF) has been linked to lactic acid bacteria survival under acidic conditions (Renault *et al.*, 1988; Poolman *et al.*, 1991; García *et al.*, 1992; Sheng and Marquis, 2007). In MLF, L-malate is decarboxylated in the cytoplasm by the malolactic enzyme to produce L-lactate and CO_2 (Renault *et al.*, 1988). The decarboxylation reaction consumes one H^+ thus contributing to the alkalization of the cytoplasm and allows ATP generation through H^+-ATPase (Poolman *et al.*, 1991). The electrogenic potential created by lactate efflux through a malate/lactate antiporter, whose gene is commonly organized in an operon structure with that of the malolactic

enzyme, may also facilitate energy production (Poolman *et al.*, 1991). Malate addition has been shown to enhance the survival of *Lactobacillus plantarum* and *Streptococcus mutans* during acid challenge at low pH values (García *et al.*, 1992; Sheng and Marquis, 2007).

5.1.5. *Alteration of metabolic pathways: energy production and conversion* (Figure 6, step 6). As acidification progresses, more energy is required to maintain pH homeostasis and as a consequence, there is a progressive reduction in the energy available for biomass synthesis until growth is eventually arrested (Cotter and Hill, 2003). In *Lactobacillus rhamnosus* GG lactate dehydrogenase (Ldh) was found to be less abundant and pyruvate dehydrogenase complex E2 component (PdhC) more abundant when grown at pH 4.8 relative to pH 5.8, suggesting a shift of pyruvate metabolism to the formation of acetil-CoA and not to lactate (Koponen *et al.*, 2012). The same change in pyruvate metabolism was observed in *Lactobacillus bulgaricus* during adaptation to pH 4.9 (Fernandez *et al.*, 2008). Fernandez et al. suggested that pyruvate was rerouted to favor FA biosynthesis in order to change membrane fluidity in respond to acid stress (Fernandez *et al.*, 2008). In agreement with this hypothesis, transcriptome results showed that FA biosynthesis was up-regulated in *L. rhamnosus* GG at pH 4.8 (Koponen *et al.*, 2012). Changes in pyruvate metabolism were also observed in *Staphylococcus aureus* during acid stress (Rode *et al.*, 2010).

L. casei BL23 acid stress response will be addressed in Chapter 3 of this PhD thesis.

5.2. Responses to stress by antimicrobial peptides.

Antimicrobial peptides (AMPs) can be divided in two classes: nonribosomally synthesized peptides, such as the gramicidins, polymyxins, bacitracins, glycopeptides, etc., and ribosomally synthesized peptides. The components of the first group are often drastically modified and are largely

produced by bacteria, whereas producers of ribosomally synthesized peptides can be found in the three domains of life. In pluricellular eukaryotes they constitute a major component of the host defense molecules of these species (Hancock and Chapple, 1999).

AMPs are constituted by 12-50 amino acids with a net positive charge of +2 to +7, thus they are usually referred to as cationic AMPs (CAMPs). Nevertheless, there also exists AMPs with anionic charge, like actagardine and mersacidine, both lantibiotics with a net negative charge of -1 (based on the BACTIBASE: database dedicated to bacteriocins (http://bactibase.pfba-lab-tun.org/)). AMPs are amphipathic molecules so that they can selectively interact with the negatively charged bacterial membranes.

Some CAMPs from the innate immune system that are present in the gastrointestinal tract are defensins (α and β) and cathelicidins; together with other immune system effectors, they constitute the first line of innate host defenses in the mucosal surfaces being some of them constitutively produced with a general surveillance like role (Hancock and Diamond, 2000; Cunliffe, 2003; Dommett *et al.*, 2005).

The gene-encoded and ribosomally synthesized, small, heat stable AMPs produced mainly by Firmicutes bacteria are referred to as ***bacteriocins***. A class of bacteriocins are the heavily modified lantibiotics, that were named after their characteristic lanthionine or methyllanthionine residues. Their structure can be either elongated, for example in nisin (Figure 7) or subtilin, or globular, as is the case for mersacidin or actagardine (Bierbaum and Sahl, 2009). Non-lantibiotic bacteriocins are similar in size to lantibiotics (<10 kDa), but are not as extensively modified (Cotter *et al.*, 2005). Bacteriocins can have a broad or a narrow spectrum of action and the producer strain utilize specific immune mechanisms against them (Cotter *et al.*, 2005). Many lactic acid bacteria produce bacteriocins (Klaenhammer, 1993; Nes *et al.*, 1996) but it is not the case of *L. casei BL23*. Bacteriocins produced by lactic acid bacteria have some desirable properties, among others, they are not toxic to eukaryotic cells, they are usually pH and heat tolerant, and they are inactivated by digestive proteases so they do not influence the gut microbiota balance (Gálvez *et al.*,

2007). In a world where the consumers demand more “healthy and natural” foods, bacteriocins produced by lactic acid bacteria that are GRAS organisms attract the interest of the food industry since they can be used as natural preservatives (Cotter *et al.*, 2005; Gálvez *et al.*, 2007). One example of this is nisin, a lantibiotic bacteriocin produced by *Lactococcus lactis* that is commonly used as a food preservative (number E234 in the European food additive list (European Economic Community, 1983)) mainly in cheese production. Nisin presents a dual mode of antimicrobial activity: it binds with high affinity to the sugar-pyrophosphate moiety of the bacterial cell-wall precursor lipid II and uses it as a docking molecule to cause inhibition of cell wall biosynthesis as well as pore formation in bacterial membranes (Figure 8) (Brötz *et al.*, 1998; Breukink *et al.*, 1999; Wiedemann *et al.*, 2001). This leads to the dissipation of membrane potential and the efflux of small metabolites from the target cell.

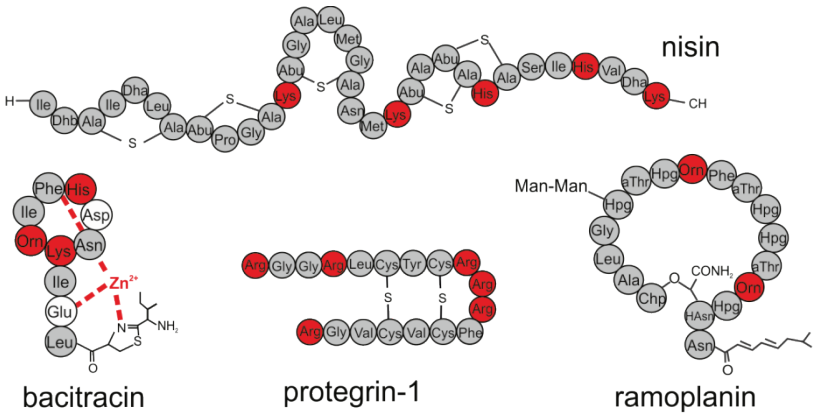


Figure 7. Structural and compositional diversity of antimicrobial peptides. Schematic representations of the structures of nisin, bacitracin, ramoplanin and protegrin. The amino acids are represented by labeled gray circles. Positively and negatively charged amino acids are highlighted red and white, respectively. Abu, aminobutyric acid; Chp, 3-chloro-4-hydroxyphenylglycine; Dha, didehydroalanine; Dhb, 2,3-didehydrobutyrine; HAsn, β-hydroxyasparagine; Hpg, hydroxyphenylglycine; Man, mannose; Orn, ornithine; aThr, allo-threonine. From *Defence against antimicrobial peptides: different strategies in Firmicutes* (Revilla-Guarinos *et al.*, 2014).

Many bacteria also produce non-ribosomally synthesized peptides such as the small circular metallo-peptide bacitracin (Figure 7) (Johnson *et al.*, 1945; Economou *et al.*, 2013), or lipodepsipeptides such as ramoplanin (Figure 7) or enduracidin (Fang *et al.*, 2006).

One important mode-of-action of the AMPs is the inhibition of cell wall synthesis (Figure 4), although additional activities have been described for some compounds, e.g. pore-formation by nisin-type lantibiotics (Figure 8) (Schneider and Sahl, 2010; Scherer *et al.*, 2013) or disturbance of membrane function by bacitracin (Figure 4 and Figure 9)) (Ming and Epperson, 2002; Schneider and Sahl, 2010; Economou *et al.*, 2013).

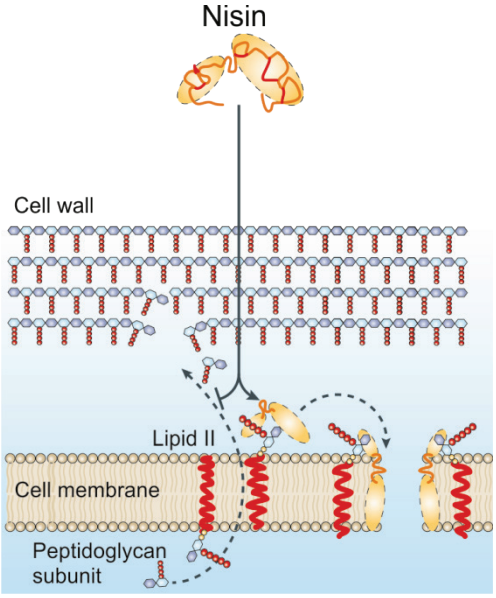


Figure 8. Mode of action of nisin. Nisin has a dual mode of action: it binds to lipid II, the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, and therefore prevents correct cell wall synthesis, leading to cell death. Nisin also use lipid II as a docking molecule to initiate membrane insertion and pore formation which leads to rapid cell death. Adapted from *Bacteriocins: developing innate immunity for food (Cotter et al., 2005).*

Different microbial responses held against CAMPs will be further described below. Some of them alter the properties of the bacterial cell envelope making it less accessible to CAMPs, for instance, changes in net negative surface charge to diminish the access of the positively charged

peptides to their surface targets. Others actively remove the peptides from their sites of action. Induction of a "bacterial dormant state" has also been described.

L. casei BL23 AMP response will be address in Chapter 2 of this PhD thesis.

5.2.1. Removal of the peptides from their site of action by ABC transporters

ABC transporters structure was described in *section 3.2.2. ATP binding cassette (ABC) transporters.*

Three main groups of AMP resistance transporters are found in Firmicutes: LanFEG, BcrAB and BceAB (reviewed in (Gebhard, 2012)). Their main characteristics are summarized in Table 3.

ABC transporters are thought to remove the peptides from their site of action. However, it is difficult to envision how a transporter could confer resistance against antibiotics that binds molecules on the cell surface. Two mechanisms of AMP detoxification have been proposed (reviewed in (Gebhard, 2012; Revilla-Guarinos *et al.*, 2014). In the first one, proposed for the LanFEG and BcrAB-type transporters, the ABC is sufficient for the transport process but additional proteins help to provide full AMP resistance. In the second one, present in the BceAB, the ABC would play a role in sensing, signaling and detoxification of the AMPs.

To the best of our knowledge, there are no studies concerning these types of transporters removing AMPs in *L. casei*, besides the data presented in this PhD thesis concerning Bce-like transporters. Nonetheless, a general overview will be presented.

Table 3: Summary of ABC transporters main characteristics. Based on (Gebhard, 2012).

Feature	LanFEG	BcrAB	BceAB
Physiological role	Mostly involved in self-protection of lantibiotic producing strains (some are genetically associated with lantibiotic biosynthesis genes). Rarely, AMP resistance in non-producing strains.	Resistance against the cyclic AMP bacitracin in producing (self-protection) and non-producing strains	AMP resistance in non-producing strains
Domain architecture	Permeases of 200–250 aa[a] and six TM[a] helices each	Permeases of approximately 230 aa with six predicted TM helices	Permease of approximately 650 aa and 10 TM helices, with a large –approx. 200 aa- extracellular domain located between helices VII and VIII
Associated proteins	Immunity proteins: LanI-type proteins (tethered to the membrane surface via an N-terminal lipoprotein anchor) and LanH-type proteins (contain three TM helices with the N-terminus located intracellularly)	Undecaprenyl-pyrophosphatase (UppP)	BceRS-like TCS[a]

Table 3: Summary of ABC transporters main characteristics. *(Continuation).*

Feature	LanFEG	BcrAB	BceAB
Regulation [b]	Mostly regulated by a TCS with prototypical periplasmic sensing HK[a,c] and OmpR family RR[a]. Others by XRE family transcriptional regulators	Mostly regulated by a TCS with IM-HK[a, c] and OmpR family RR. Others by XRE transcriptional regulators	BceRS-like TCS with IM-HK and OmpR family RR. Transporter regulating its own expression in response to CAMPs (see text for details)
Direction of substrate transport	Export (the lantibiotic is removed from the cytoplasmic membrane to the culture supernatant)[d]	Unknown (export postulated)[e]	Unknown. Import suggested, followed by cytoplasmic enzymatic inactivation of the CAMP through degradation[e]
Substrates	Lantibiotics (nis, gall, epi, nuk, sub, etc.)[f] and dipeptide lantibiotics (lact)[f]	Cyclic AMP: bac[f]	Lantibiotics (nis, sub, gall, mer)[f], cyclic AMPs (bac), lipodepsipeptides (end)[f], glycopeptides (van, tei)[f], peptides from the immune system of higher organisms like cathelicidines (LL-37, ind, ovi)[f], and defensins (hBD3, bre, ple)[f]

[a] *aa:* amino acids; *TM:* transmembrane; *TCS:* two component systems; *HK:* Histidine kinase; *RR:* Response regulator; *IM-HK:* intramembrane-sensing histidine kinase.
[b] (Gebhard, 2012); [c] (Mascher *et al.*, 2006); [d] (Otto *et al.*, 1998; Stein *et al.*, 2003; Okuda *et al.*, 2008); [e] (Rietkötter *et al.*, 2008; Hiron *et al.*, 2011).
[f] *act*: actagardine; *bac*: bacitracin; *bre*: brevinin; *end*: enduracidin; *epi*: epidermin; *gall*: gallidermin; *ind*: indolicidin; *lact*: lacticin 3147; *mer*: mersacidin; *nis*: nisin; *nuk*: nukacin; *ovi*: ovispirin; *ple*: plectasin; *sub*: subtilin; *tei*: teicoplanin; *van*: vancomycin.

5.2.1.1. LanFEG and BcrAB type transporters Most LanFEG-type transporters are involved in self-protection in lantibiotic producer strains, and recognize only a narrow range of related substrates (Table 3) (Otto *et al.*, 1998; Stein *et al.*, 2003; Gebhard, 2012). BcrAB transporters mediate resistance against bacitracin (Podlesek *et al.*, 1995; Neumüller *et al.*, 2001). LanFEG and BcrAB transporters are composed of two permease subunits with six predicted transmembrane helices, which can be encoded by two separate genes (*lanE* and *lanG* in LanFEG-type) or a single gene (*bcrB* in BcrAB-type). The ATPase subunits are encoded by separate genes in both types of transporters (*lanF* and *bcrA*, respectively) (Gebhard, 2012). Phylogenetic analyses have shown that BcrAB and LanFEG are closely related, and they also share functional characteristics (Gebhard, 2012).

Several studies reported that these transporters remove lantibiotics from the cytoplasmic membrane and discharge them to the extracellular medium (Figure 9, step 3) (Stein *et al.*, 2003; Stein *et al.*, 2005; Okuda *et al.*, 2008). It remains unclear, however, how cells prevent CAMPs from binding again to the cytoplasmic membrane. In this regard, the high degree of co-occurrence of LanFEG-type transporters with LanI or LanH immunity proteins (78%), and of BcrAB-type transporters with UppP (undecaprenyl pyrophosphate phosphatase)-encoding genes (77%) should be noted (Gebhard, 2012). To date, conflicting data is reported on whether transporters and immunity proteins act cooperatively or independently of each other to confer resistance.

An independent action has been proposed for the nisin resistance system of *Lactococcus lactis*, constituted by the immunity protein NisI and the transporter NisFEG and for the SpaI-SpaFEG system of *Bacillus subtilis*, which provides self-protection against subtilin (Stein *et al.*, 2003; Stein *et al.*, 2005). Other studies suggested cooperativity between NisI and NisFEG (Ra *et al.*, 1999; Takala *et al.*, 2004; Takala and Saris, 2006), or between the nukacin ISK-1 immunity protein NukH and NukFEG (Okuda *et al.*, 2008).

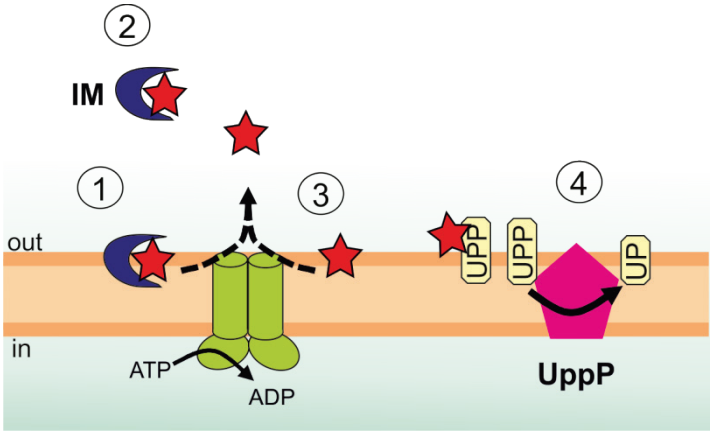


Figure 9. Schematic representation of the postulated models of action of LanFEG and BcrAB transporters conferring CAMP resistance. Transporters are shown in green, and ATP-hydrolysis and substrate translocation are indicated by black solid and dashed arrows, respectively. CAMPs are shown as red stars. 1) Transport assisted by NukH-type immunity proteins. 2) Binding of CAMPs by NisI-type immunity proteins. 3) Hydrophobic vacuum-cleaner model of efflux mechanism. 4) CAMPs (bacitracin) bind to the pyrophosphate group of UPP preventing its dephosphorylation by undecaprenyl pyrophosphate phosphatase (UppP; pink pentagon); UPP and UP molecules are shown schematically and dephosphorylation is indicated by a black arrow. IM, immunity protein. From *Defence against antimicrobial peptides: different strategies in Firmicutes* (Revilla-Guarinos *et al.*, 2014).

The LanI-type immunity protein NisI can be found as a membrane-anchored protein and as a lipid-free form, being the later also able to bind nisin (Stein *et al.*, 2003; Koponen *et al.*, 2004; Takala *et al.*, 2004). Takala *et al*. proposed that the NisFEG complex exports membrane bound nisin back to the culture supernatant creating a high local concentration of nisin where it could be intercepted by lipid-free NisI, which would move nisin away from the cell surface avoiding its re-association with its cellular targets (Figure 9, steps 2 and 3) (Takala *et al.*, 2004).

In the case of the LanH-type immunity protein NukH and NukFEG, the proposed mechanism implies that NukH functions as a substrate-binding protein for NukFEG transporter. NukH binds nukacin ISK1 in an energy-independent manner and this complex is transported to the extracellular space

by NukFEG in an energy-dependent manner (Figure 9, step 1) (Okuda *et al.*, 2008).

It is attractive to postulate a concerted action of transporters and immunity proteins, which might explain the mechanism of resistance: the transporter would remove cell membrane-associated CAMPs and release them to the external media while immunity proteins would bind and sequester the CAMPs, thus avoiding re-association with the bacterial surface (Fig. 9, steps 1, 2 and 3) (Takala *et al.*, 2004).

BcrAB-type transporters are often encoded in an operon with a UppP encoding gene (Gebhard, 2012). It is therefore likely that the bacitracin resistance mechanism of the transporter is tightly linked to UppP activity (Figure 9, steps 3 and 4).

The carrier lipid UP is essential for the biosynthesis of several cell surface polymers. UPP is the precursor of UP, and has to be dephosphorylated to increase the availability of the lipid carrier UP for biosynthetic processes (Figure 4). The dephosphorylation is carried out by the UppPs (Figure 9, step 4) (Barreteau *et al.*, 2009). Bacitracin binds to UPP (Figure 4 and Figure 9, step 4) but has less affinity for monophosphates (Storm and Strominger, 1973). In fact, it has been shown that increasing UppP activity confers increased resistance to bacitracin (Bernard *et al.*, 2005; Shaaly *et al.*, 2013), whereas its inactivation led to increased sensitivity (Cao and Helmann, 2002; Shaaly *et al.*, 2013). Therefore, maximal protection is most likely ensured when transporter and UppP act concertedly (Podlesek *et al.*, 1995). The resistance mechanism to bacitracin could be as follows: upon induction of the operon encoding the BcrAB transporter and the Upp, the former would decrease the bacitracin associated to the cell surface and the later would decrease the bacitracin cell surface target, avoiding re-association of the expelled bacitracin and resulting in higher bacitracin resistance (Figure 9, steps 3 and 4).

The efflux mechanism used by these transporters still awaits elucidation although the hydrophobic vacuum-cleaner model (Figure 9, step 3), originally proposed for the eukaryotic P-glycoprotein, a multidrug ABC transporter (Raviv *et al.*, 1990), currently receives major acceptance. This model hypothesizes

that the target compounds enter the transporter binding sites directly from the membrane and are released to the extracellular medium. Transport from the inner leaflet to the extracellular medium was demonstrated for the *L. lactis* multidrug resistance ABC transporter LmrA (Bolhuis *et al.*, 1996). However, it remains to be seen if such a mechanism is directly applicable to the CAMP transporters discussed here, whose substrates are most likely located in the outer leaflet of the membrane.

5.2.1.2. BceAB like transporters

These ABC transporters appear associated to BceRS like TCS. A detailed description of their working mechanism can be found in section 6.1. *Bce like two component systems*. A summary of their characteristics is presented in T*able 3.*

5.2.2. Modification of the cell surface properties

The net negative charge of the bacterial cell surface is exploited by CAMPs in their antimicrobial action (Peschel, 2002; Peschel and Sahl, 2006). As was previously mentioned when talking about the Dlt system (see section *3.1.2.1 Dlt system*) studies of *dlt* mutants have shown that D-alanylation of TAs has a wide range of physiological consequences in different bacteria as well as in their interactions with other organisms (Neuhaus and Baddiley, 2003; Weidenmaier and Peschel, 2008; Swoboda *et al.*, 2010). Between these consequences, the role of D-alanylation on the resistance against CAMPs (Figure 11), has been documented by numerous studies (Davie and Brock, 1966; Peschel *et al.*, 1999; Boyd *et al.*, 2000; Abachin *et al.*, 2002; Poyart *et al.*, 2003; Kristian *et al.*, 2005; Fabretti *et al.*, 2006; Kovács *et al.*, 2006; Walter *et al.*, 2007; Saar-Dover *et al.*, 2012). In the same way, although MprF mutants defective in L-lysynilation of phospholipids (see section *3.2.1. MprF protein*) show little phenotypic changes under standard laboratory conditions, they are strongly susceptible to CAMPs (Thedieck *et al.*, 2006; Ernst and Peschel, 2011). These observations have been explained by postulating that D-alanylation of TAs and L-lysynilation of phospholipids would diminish the electrostatic

attraction between CAMPs and the cell envelope by reducing the net charge of the cell surface (Figure 10 and 11B) (Peschel *et al.*, 1999; Peschel, 2002; Neuhaus and Baddiley, 2003; Peschel and Sahl, 2006; Swoboda *et al.*, 2010; Matsuo *et al.*, 2011; Anaya-López *et al.*, 2013). This model is in accordance with different experimental observations demonstrating that a lack of alanylation leads to increased binding of several positively charged molecules such as Mg^{2+} (Heptinstall *et al.*, 1970) or Cyt *c* (Wecke *et al.*, 1997; Peschel *et al.*, 1999; Kristian *et al.*, 2005; Saar-Dover *et al.*, 2012) and also the CAMPs gallidermin (Peschel *et al.*, 1999) and vancomycin (Peschel *et al.*, 2000).

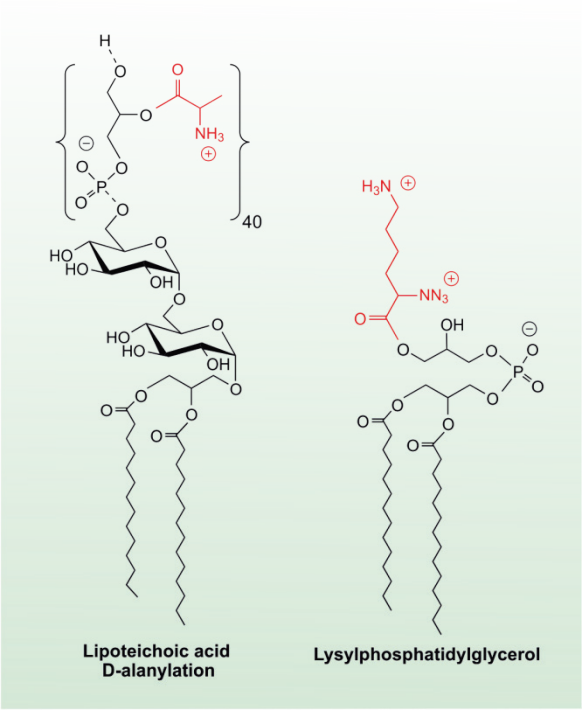


Figure 10. Modification of the bacterial cell envelope components involved in CAMP resistance. D-alanylation of teichoic acids (left) and L-lysynilation of phosphatidylglycerol (right). Alanine and lysine residues are shown in red. Adapted from *How do bacteria resist human antimicrobial peptides?* (Peschel, 2002).

However new experimental evidences led to the proposal of new models, mainly for the action of Dlt-system, that will be exposed in more detail below (reviewed in (Revilla-Guarinos *et al.*, 2014)).

A *dltA* mutant of *Streptococcus agalactiae* was shown to bind three times more Cyt *c* than the wild-type strain. However, no significant differences in binding of a number of CAMPs were detected, indicating that different interactions account for the binding of Cyt *c* and the binding of CAMPs (Saar-Dover *et al.*, 2012). A direct estimation of the net electric charge of *Lactococcus lactis* cells by electrophoretic mobility measurements detected no significant difference in global cell charge between the wild-type strain and a *dltD*-defective mutant (Giaouris *et al.*, 2008). The estimation of cell electric charge by binding assays relies on the assumption that the interaction between the cell envelope and the ligand is essentially electrostatic and independent of the nature of the ligand. However, the contribution of other interactions should be taken into account. For example, hydrophobic interactions between Cyt *c* and cell membrane lipids have been observed earlier (Rytömaa *et al.*, 1992; Cortese *et al.*, 1995) and might influence the binding affinity for Cyt *c* of the bacterial cell envelope.

Based on these and the following observations, an alternative model (Figure 11C) was recently proposed that suggests that D-alanylation of TAs leads to structural modifications of the cell wall making it more compact and less permeable and hence restricting the access of CAMPs to the membrane (Saar-Dover *et al.*, 2012). In support, these authors showed that the cell wall of a *S. agalactiae dltA* mutant is less dense and its surface is less rigid than that of the wild-type strain. It was shown that binding of CAMPs to LTA was not significantly different between the two strains; however, access of CAMPs to the membrane was increased in the *dltA*-defective mutant. Furthermore, the authors observed that high NaCl concentration reduced the penetration of CAMPs through the cell wall of the *dltA* strain to restore wild-type behavior.

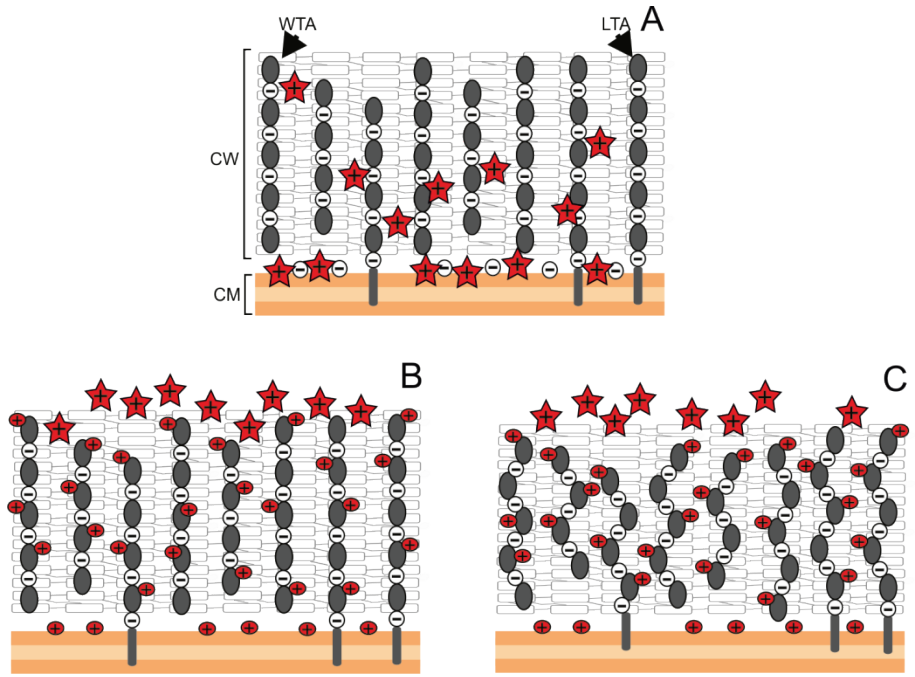


Figure 11. Models of the effect of changes in the bacterial cell surface in CAMPs resistance. From *Defence against antimicrobial peptides: different strategies in Firmicutes* (Revilla-Guarinos *et al.*, 2014). CM, cytoplasmic membrane; CW, cell wall; WTA, wall teichoic acids; LTA, lipoteichoic acids. CAMPs are depicted as red stars.
A, cell envelope in the absence of D-alanylation of TAs and L-lysinilation of membrane phospholipids. Local concentration of CAMPs is increased presumably by electrostatic interactions with the cell envelope. CAMPs can reach the cell membrane and interact with their targets.
B, electrostatic hindrance model for CAMP resistance. D-alanylation of TAs and L-lysinylation of membrane phospholipids decrease the net negative charge of the cell envelope and the local concentration of CAMPs.
C, electrostatic and steric hindrance model. D-alanylation of TAs modifies the cell wall structure making it less permeable to CAMPs.

Previous studies had already noted alterations in the cell wall structure in response to the extent of D-alanylation of TAs. Ou and Marquis observed that removal of D-alanyl esters from TAs of *S. aureus* caused an expansion of the cell wall (Ou and Marquis, 1970). Furthermore, it is well established that TAs play a major role in the structure of the cell wall and that the ionic

environment is a determinant in the structural transitions of TAs (Doyle *et al.*, 1974; Pal *et al.*, 1990). Incorporation of D-alanyl residues in TAs would change the ionic environment around TAs, thus modulating the conformational transitions of TAs (Neuhaus and Baddiley, 2003; Saar-Dover *et al.*, 2012). These transitions could account for the structural differences observed between the cell walls of D-alanyl-TAs deficient strains and those of the parental strains.

Taken together, this evidence supports the idea that D-alanylation of teichoic acids modifies the electrostatic interactions between TAs leading to a strengthening of the cell wall and an increase of its barrier properties (Figure 11C). This would impede the access of the usually amphipathic CAMPs to the membrane.

Finally, it is worth noticing that whereas ABC transporters usually confer resistance against a small number of compounds, induction of *dlt* and *mprF* genes elicits a more general resistance against CAMPs.

5.2.3. Biofilms as antibiotic induced resistance mechanisms.

Planktonic cells are single cells that may swim or float in a liquid medium (Hall-Stoodley *et al.*, 2004). In response to different factors, planktonic cells can associate and stick to each other on a surface forming a biofilm. These adhered groups of microorganisms are usually embedded within a self-produced matrix of extracellular polymeric substance (composed of extracellular DNA, proteins and polysaccharides).

Microbial communities forming a biofilm present higher resistance to environmental stress than planktonic cells and lower sensitivity to antibiotics: 10 to 1000 times the antibiotic concentration needed to kill planktonic cells is required for killing biofilms (Mah and O'Toole, 2001; Hall-Stoodley *et al.*, 2004). Furthermore, subinhibitory concentrations of antibiotics induce bacterial biofilm formation *in vitro*, both in Gram positive and Gram negative microorganisms (Kaplan, 2011). As a consequence of this findings the idea emerges that antibiotics are bacterial weapons at high concentrations for

fighting competitors; whereas at the subinhibitory concentrations they are usually found in microbial ecosystems or during the different stages of clinical treatments, antibiotics constitute signaling molecules (intra and inter specific) that may regulate the homeostasis of microbial communities (Kaplan, 2011; Liu *et al.*, 2013). Therefore, it has been suggested that biofilm formation upon exposure to subinhibitory concentrations of antibiotics may represent and inducible mechanism of antibiotic resistance (Kaplan, 2011). This antimicrobial-induced biofilm formation would account for the difficulties to eradicate recurrent bacterial infections in clinic diseases (Berditsch *et al.*, 2012; Kaplan *et al.*, 2012; Abdelhady *et al.*, 2013; Gomes *et al.*, 2013).

L. casei can be found constituting multispecies biofilms, for example in the oral cavity (Modesto *et al.*, 2000; Quevedo *et al.*, 2011; Arthur *et al.*, 2013). Interestingly, Palomino et al. (Palomino *et al.*, 2013) reported that *L. casei* BL23 has an increased ability to form biofilms and to bind cations in high-salt conditions and this behavior correlated with modifications of surface properties involving TA. They also showed that, in these high-salt conditions, *L. casei* BL23 produces less LTA, and that this anionic polymer has a shorter average chain length and a lower level of D-alanyl-substitution. Accordingly, the transcript levels of the *dltABCD* operon showed a 16-fold reduction (Palomino *et al.*, 2013). However, to the best of our knowledge, no studies addressing the effect of AMPs in *L. casei* biofilm formation have been reported.

6. SIGNAL TRANSDUCTION: TWO COMPONENT SYSTEMS

Two component systems (TCS) play a key role in the detection of environmental changes and regulation of the response to those changes (Grebe and Stock, 1999; Mascher *et al.*, 2006; Jordan *et al.*, 2008; Gao and Stock, 2009; Jung *et al.*, 2012; Schrecke K. *et al.*, 2012). TCS genes are found in all three domains of life, being considerably less abundant in archaea and eukaryotes and most abundant in the genomes of Gram-negative bacteria and

Cyanobacteria (Capra and Laub, 2012). Bacterial species contain on average about two dozen TCS regulating most aspects of bacterial physiology, like chemotaxis, nutrient uptake, stress response, central metabolism and virulence (Barakat *et al.*, 2011). The patterns of TCS gene content strongly suggest that organisms expand their set of two-component signaling genes to adapt to fluctuations in their environment (Capra and Laub, 2012). In that way, bacteria that live in constant environments typically encode relatively few TCS genes, and, many obligate intracellular parasites and endosymbionts harbor only a few pathways or sometimes none at all. On the contrary, bacteria that inhabit rapidly changing or diverse environments typically encode large numbers of these signaling proteins, like *Myxococcus xanthus*, with 136 histidine kinases and 127 response regulators and *Nostoc punctiforme*, with 160 kinases and 98 regulators (reviewed in *Evolution of Two-Component Signal Transduction Systems* (Capra and Laub, 2012)).

TCS are typically constituted by a membrane histidine kinase (HK) that acts as a signal sensor/transducer and a response regulator (RR) that usually acts as a transcriptional activator/repressor (Stock *et al.*, 2000; Gao and Stock, 2009). Both proteins have a modular structure: HKs usually have two modules involved in the phosphorylation reaction, the kinase and H-box domains, and usually an N-terminal transmembrane sensory domain that monitors environmental signals. On the other hand, the RR has a receptor domain of the phosphoryl group and a C-terminal effector domain. The domains involved in the phosphorylation reaction of both HKs and RRs are homologous in all TCS (Koretke *et al.*, 2000) whereas the sensor and effector domains are characteristic of individual TCS and determine their specificity.

HKs exert both positive and negative control over the activity of the RRs (Huynh and Stewart, 2011). In the positive control, usually in response to a stimulus, HKs autophosphorylate at a histidine residue (H-box), and the high-energy phosphate group is subsequently transferred to an aspartyl residue on the RR receptor domain. Phosphorylation of the RR in turn modulates the activity of the RR effector domain (Gao and Stock, 2009). On the contrary, in the negative control the HK transmitter domain mediates the RR receiver domain dephosphorilation, termed HK phosphatase activity, enhancing in that

way the intrinsic RR receiver autodephosphorylation rate (Huynh and Stewart, 2011). The positive and negative controls determine the output response. Functions of the negative control are to reset the baseline signaling state rapidly upon stimulus depletion and also to suppress cross-talk resulting from unwanted phosphorylation of the RR by non-cognate HKs (Huynh and Stewart, 2011).

A three step strategy can be followed when starting the study of the TCS present in one microorganism (Fabret *et al.*, 1999; Hancock and Perego, 2004; Biswas *et al.*, 2008). Firstly, sequence analysis of the complete genome (if available) looking for sequences encoding for TCS. The structural and functional principles for these bioinformatic analyses can be based on the conserved ATP-binding site and conserved histidine motif of sensor HK, and the aspartate domains of RRs. Secondly, comparative analyses with previously characterized TCS present in other microorganisms should be performed to assign putative functions to the newly identified TCS. Finally, construction of mutants in each TCS and phenotypic characterization should be performed, to precisely assign a biological function. A deeper knowledge on TCS can be obtained for example by methods that allow to monitor protein phosphorylation and dephosphorylation events, structural and functional characterization of RR activation, determination of the cellular localization of TCS proteins, bacterial two hybrid assays to determine interaction of TCS with other cellular proteins, among others (Scharf, 2010; Falord *et al.*, 2012; Kallenberg *et al.*, 2013).

A major part of this PhD thesis will be focus on two TCS from *Lactobacillus casei* BL23: TCS09 and TCS12. TCS09 and TCS12 are orthologs to the extensively studied BceRS TCS of *Bacillus subtilis* (Ohki *et al.*, 2003; Bernard *et al.*, 2007; Rietkötter *et al.*, 2008; Staroń *et al.*, 2011; Kallenberg *et al.*, 2013) which names a group of TCS sharing structural and mechanistic characteristics, the Bce-like two component systems (Dintner *et al.*, 2011). This group will be described in further detail in the next section.

6.1 Bce-like two component systems

BceRS-like TCS are composed of an intramembrane-sensing histidine kinase (IM-HK) (Mascher, 2006), BceS-like, and an OmpR family RR , BceR-like (Figure 12).

IM-HKs possess two transmembrane helices with a short (less than 20 amino acids) loop between them in the N-terminal sensing domain, and a cytoplasmic transmitter domain with only the DHp (dimerization histidine phosphotransfer) and catalytic ATPase domains. The absence of extracytoplasmic domains and any additional domains that could allow signal detection within the cytoplasm, led to the suggestion that IM-HKs sensed their stimulus in the surface or within the cytoplasmic membrane (Mascher, 2006). BceR contains a winged helix–turn–helix DNA-binding output domain. BceRS-like TCS appear genetically and functionally associated with BceAB-like ABC transporters (Table 3) consisting of a nucleotide-binding domain (NBD) subunit BceA (ATPase) and a membrane spanning domain (MSD) subunit BceB (permease) (Dintner *et al.*, 2011; Gebhard and Mascher, 2011; Gebhard, 2012). These BceRSAB-like modules (named after the bacitracin resistance module BceRSAB of *Bacillus subtilis* (Mascher *et al*., 2003; Ohki *et al.,* 2003)) are antimicrobial peptide detoxification systems in non-producing strains, where the ABC plays a dual role: it mediates AMP resistance/detoxification (output) and it is required for AMP sensing (input).

Several Bce-like detoxification modules have been characterized in *Bacillus subtilis, Bacillus licheniformis, Lactococcus lactis, Listeria monocytogenes, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae, Streptococcus mutans* and *Streptococcus salivarius* (reviewed in (Dintner *et al.*, 2011; Gebhard and Mascher, 2011; Gebhard, 2012; Schrecke K. *et al.*, 2012; Revilla-Guarinos *et al.*, 2014)). However, Bce-like detoxification modules have not been characterized in *Lactobacillus* yet.

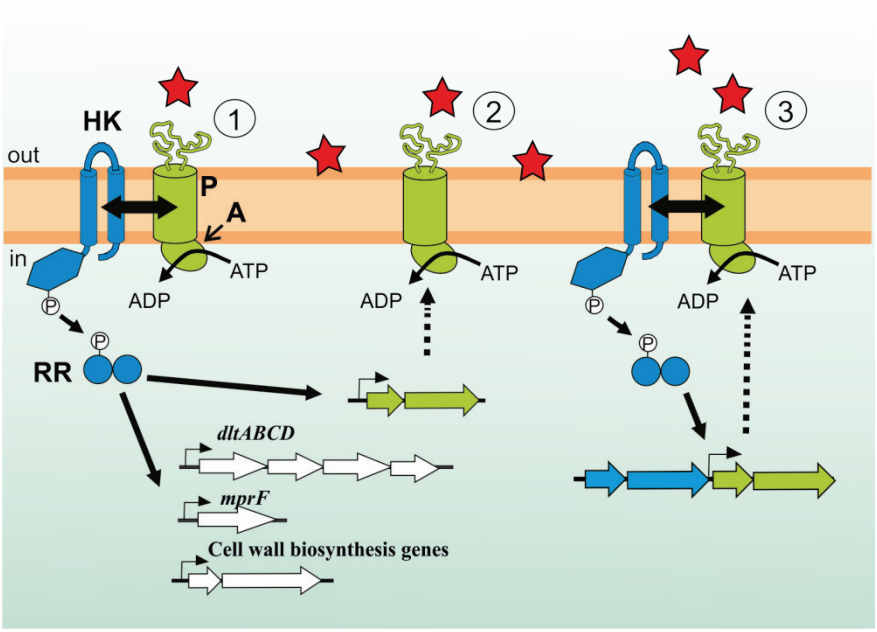


Figure 12. Schematic representation of the postulated models of BceRSAB-type modules conferring CAMP resistance. 1) Sensing, 2) resistance and 3) dual function BceAB-like transport systems. ATP-hydrolysis is indicated by black solid arrows. CAMPs are shown as red stars. Signaling between the transporters (green) and the TCS (blue) is indicated by a double-headed black arrow. Phosphotransfer within TCS and gene activation are indicated by black arrows, and the increased expression of transporter genes is indicated by straight dotted arrows. The positions of promoters relative to genes were chosen arbitrarily. Likely dimerization of BceB-type permease subunits is not shown for reasons of simplicity. HK, histidine kinase; RR, response regulator; A, ATPase; P, permease. From *Defence against antimicrobial peptides: different strategies in Firmicutes*(Revilla-Guarinos *et al.*, 2014).

How is the stimuli sensed by Bce-like systems?

Bce-like modules are characterized by being able to recognize structurally different substrates while at the same time being able to distinguish between similar ones (Gebhard and Mascher, 2011; Staroń *et al.*, 2011). The molecular mechanism behind this characteristic is still unclear. Furthermore, it has been shown that Bce-like systems do not detoxify all the AMPs that induce their expression (Staroń *et al.*, 2011).

Generally, the HK of a TCS mediates stimulus perception (Mascher *et al.*, 2006). However, in the Bce-like systems it has been shown that the BceS-like HKs are not the sensors of the stimulus by itself, but instead depend on the ATP driven transport of the substrate by the cognate ABC transporter for signal transduction (Bernard *et al.*, 2007; Meehl *et al.*, 2007; Rietkötter *et al.*, 2008; Hiron *et al.*, 2011).

BceB-like permeases are membrane proteins (of approximately 650 amino acids) with ten transmembrane helices and a large extracytoplasmic domain (ECD) of approximately 200 amino acids between helices 7 and 8. While phylogenetic analyses of the transmembrane regions of BceB-like transport permeases showed good sequence conservation at the amino acid level, the ECD regions did not (Dintner *et al.*, 2011). Furthermore, no correlation between ECD sequence and substrate range could be found. This high degree of variability in the ECD supports previous suggestions arguing that these regions of the permeases contain the substrate binding domain of the transporters, and that the high degree of variability is required to respond to the set of compounds that these systems mediate resistance to (Rietkötter *et al.*, 2008). Some experimental results further support this idea. In *S. aureus* ABC transporter VraDE confers bacitracin resistance while ABC transporter VraFG is involved in resistance to colistin. Domain-swapping studies by Hiron *et al*. showed that a transporter with a quimeric VraG permease harbouring the ECD of VraE, $vraFG^{*vraE}$, restored bacitracin resistance in a *ΔvraDE* mutant but was not able to restore colistin resistance in a *ΔvraFG* mutant strain (Hiron *et al.*, 2011). Furthermore, in a study by Kallenberg *et al*. a random mutagenesis approach was performed in order to identify residues in permease BceB of *B. subtilis* involved in mediating resistance to bacitracin or signaling to the BceS HK (Kallenberg *et al.*, 2013). Of all the clones obtained, the authors did not identify any mutation in the ECD, further supporting the high degree of variability that this domain can support while still retaining full activity.

The resistance mechanism is as follows: the permease subunit of the system, BceB, detects the stimulus and transfers the information to the HK BceS, which does not function as a direct sensor but rather as a signal transfer relay to BceR. Activation of BceR then induces the expression of *bceAB* and

sometimes, other target genes, which ensures antibiotic resistance. Several aspects of this resistance mechanism are worth noticing: firstly, that all four members of BceRSAB-like systems are required for AMP sensing and signaling. A comprehensive bioinformatic and phylogenetic analysis of BceRS-like TCS and BceAB-like transporters showed that they have co-evolved in Firmicutes, supporting the strong functional link between them (Dintner *et al.*, 2011). Secondly, that the modular design of these detoxification systems allows regulatory flexibility.

Direction of substrate transport

The direction of substrate transport is unknown. It has been suggested that the AMPs would be imported for further cytoplasmic enzymatic inactivation and degradation (Rietkötter *et al.*, 2008; Hiron *et al.*, 2011).

Sensing ABCs versus resistance ABCs

Different types of ABC transporters can be found in the Bce-like modules according to its function: sensing ABCs, resistance ABCs and ABCs with both functions.

Sensing ABCs (Figure 12, step 1) are those transporters that mediate the sensing of the substrate and signaling, together with their partner BceRS like TCS, but which *do not* mediate the actual resistance. Importantly, although they do not detoxify the inducing substrate, they are able to transport it since ATP hydrolysis is also required (Hiron *et al.*, 2011). It has been suggested that transport by this ABCs takes place at a low rate, sufficient for signaling the presence of the antibiotic to the partner HK but not enough for conferring resistance to it (Gebhard and Mascher, 2011). Since the sensing ABCs do not mediate the resistance, its expression does not need to increase in response to the inducing substrate (Dintner *et al.*, 2011). Another characteristic feature of BceRSAB modules harboring a sensing ABC is that they usually control a relatively large regulon that includes ABC transporters (the sensing transporter and/or associated resistance transporters), genes controlling the cell wall stress response like *dltABCD* and *mprF* and genes for cell wall biosynthesis. Some examples are described below.

S. aureus possesses two BceRSAB-like modules, GraRS-VraFG (also named ApsRS-VraFG) and BraRSDE (the latter also named NsaRSAB and BceRSAB) where the transporters of both systems are just sensing ABCs (Gebhard and Mascher, 2011; Kawada-Matsuo *et al.*, 2011). In the BraRSDE module, BraDE is a sensing ABC and resistance against bacitracin and nisin is mediated by a second ABC transporter, VraDE, not genetically associated in the genome to the Bra system but whose expression is under BraRS TCS control. BraRS also controls the expression of BraDE (Pietiäinen *et al.*, 2009; Hiron *et al.*, 2011). In the Aps/GraRS-VraFG system, VraFG is a sensing transporter and resistance involves expression of *dlt* and *mprF*, which together with VraFG, are under transcriptional control of Aps/GraRS TCS (Li *et al.*, 2007; Meehl *et al.*, 2007; Falord *et al.*, 2011; Falord *et al.*, 2012).

Resistance ABCs (Figure 12, step 2) *mediate* the actual resistance to the antibiotic, but they are not involved in peptide sensing and signaling. They are usually controlled by nongenetically associated BceRS-like TCS. That would be the case of the aforementioned VraDE transporter of *S. aureus which does not possess its own TCS and that mediates resistance to AMPs but not signaling* (Hiron *et al.*, 2011). In *L. monocytogenes* VirRS TCS regulates the expression of the nongenetically associated ABC transporter AnrAB which has also been shown to be involved in AMP resistance (Collins *et al.*, 2010).

The third group of transporters are those with a ***dual function***: they are involved in substrate sensing and signaling, but also mediate resistance to it (Figure 12, step 3). One characteristic feature of these systems is that after induction of the system upon substrate binding there is an increase in the expression of the ABC to mediate the resistance, thus the transporter regulates its own expression in response to AMPs through the action of its cognate TCS. Once the inducing compound is removed, the system switches off.

Two out of the three *Bacillus subtilis* Bce-like resistance modules possess a transporter with a dual function: BceRSAB and PsdRSAB. BceRSAB is most effective against bacitracin (Mascher *et al.*, 2003; Ohki *et al.*, 2003), but it also confers resistance to mersacidin, actagardine and plectasin (Schneider *et al.*,

2010; Staroń *et al.*, 2011). The paralogous system PsdRSAB is induced by enduracidin, actagardine, gallidermin, nisin and subtilin, and mediates resistance to all of its inducers except to actagardine (Staroń *et al.*, 2011). The features that characterize the *B. subtilis* Bce-like systems are firstly that, with the exception of some cross-phosphorylation between BceS HK to the PsdR RR (Rietkötter *et al.*, 2008), the two systems are independent detoxification modules. Secondly, that the cognate ABC transporter is the only target of regulation of the TCS in response to the inducing substrates (Ohki *et al.*, 2003; Rietkötter *et al.*, 2008; Staroń *et al.*, 2011).

It is interesting to note here that it has been recently shown that even in a dual-function transporter like BceAB of *B. subtilis*, where signaling and resistance are functionally interconnected, they are not strictly coupled processes. Random mutagenesis studies allowed to obtain BceB mutants affected in signaling but still able to confer bacitracin resistance and vice versa, mutants where signaling was maintained but resistance was completely lost (Kallenberg *et al.*, 2013). These results suggest that the current specialized sensor ABCs and resistance ABCs could have originally been dual function systems, selected in the course of evolution towards more specific and restricted functions.

How is the signal transduced?

Although some hypotheses have been proposed (reviewed in (Schrecke K. *et al.*, 2012; Revilla-Guarinos *et al.*, 2014)), the exact molecular mechanism by means of which the signal information is transferred from the ABC to the HK is not known yet. Several mechanistic ideas, experimentally proved, should be kept in mind when trying to answer this question.

(i) As described above, the ECD of the permease is required for substrate recognition and binding (Rietkötter *et al.*, 2008; Hiron *et al.*, 2011).

(ii) ATP driven transport of the substrate is required for signal transduction (Bernard *et al.*, 2007; Rietkötter *et al.*, 2008).

(iii) No cytoplasmic domains beyond DHp and catalytic ATPase domains are present in the BceS-like HKs.

(iv) The presence of additional proteins required for signaling has been so far only described in *S. aureus* (Falord *et al.*, 2012) and *S. epidermidis* (Li *et al.*, 2007).

(v) Bacterial two hybrid (B2H) assays with the GraXSR-VraFG system of *S. aureus* showed that GraS HK interacts with both GraX and the VraG permease and that GraX also interacts with VraF ATPase and GraR RR, suggesting that GraX acts as a cofactor of GraSR by signaling through GraS (Falord *et al.*, 2012). Similarly, B2H in *B. subtilis* also showed a direct interaction of BceB with BceA and BceS (Kallenberg *et al.*, 2013) .

(vi) The HK possess a short extracytoplasmic loop.

(vii) However, evidence support that the extracytoplasmic loop of the HK is also required for substrate recognition. *apsS* from *S. epidermidis* (responsive to hBD3) and *apsS* from *S. aureus* (not responsive to hBD3) show an overall similarity of 70%, whereas their extracellular loop is only 33% similar. A hybrid ApsS protein of *S. aureus* with the extracellular loop of *S. epidermidis* restored *apsS* inducibility by hBD3 in the *S. aureus apsS* mutant (Li *et al.*, 2007). In a different study of the same authors, antibodies against the short, negatively charged loop of the *ApsS* HK of *S. epidermidis* prevented up-regulation of the *dltB* target gene upon addition of the inducer AMP, suggesting that AMPs bind to the extracellular loop of ApsS (Li *et al.*, 2007).

(viii) Kallenberg *et al*. found a BceB mutation in *B. subtilis*, G215R, which completely abolished the interaction with BceS in B2H assays. However, this mutation allowed the protein to maintain a 30% of wild type signaling, indicating that the signal could still be passed to the HK (Kallenberg *et al.*, 2013).

Three hypothesis for signal transduction have been proposed (Schrecke K. *et al.*, 2012) and in light of these experimental results any of them can be directly ruled out.

It has been proposed that BceAB-type transporters might function as importers so that detection by the cognate HKs and CAMP inactivation would occur in the cytoplasm (Rietkötter *et al.*, 2008; Hiron *et al.*, 2011). However, the identification of mutations in BceB that significantly decreased signaling activity while retaining bacitracin resistance seems to rule out this hypothesis, at least for the BceRSAB module (Kallenberg *et al.*, 2013).

A second hypothesis postulates that the transporter binds the substrate and presents it to the HK, which would then only recognize it in complex with the transporter (Schrecke K. *et al.*, 2012). In this case signal detection by the HK might occur through the short extracytoplasmatic loop of the HK. This idea is supported by the results obtained with the hybrid ApsS protein of *S. aureus* with the extracellular loop of ApsS from *S. epidermidis which* restored *apsS* inducibility by hBD3 in the *S. aureus apsS* mutant (Li *et al.*, 2007). But this hypothesis does not explain why ATP hydrolysis by the transporter is required for signal transfer, since substrate binding should be ATP-independent.

The third hypothesis postulates that signal transfer occurs by direct protein-protein contact between the ABC transporter and the HK, where a conformational change in the transporter due to substrate binding and transport could activate the HK. This hypothesis is supported by the results obtained by B2H carried out with the GraXSR-VraFG system of *S. aureus* (Falord *et al.*, 2012) and the BceRSAB of *B. subtilis* (Kallenberg *et al.*, 2013) which revealed interactions between HKs and cognate ABC transporters.

BceR-like RR target genes

It has been described that the consensus binding sequence for BceR-like regulators is TNACA-N_4-TGTAA, being N_4 an AT-rich spacer (de Been *et al.*, 2008; Dintner *et al.*, 2011).

Although, one of the main target genes of this resistance modules are ABC transporters (the TCS associated ABC and/or additional resistance ABC transporters), as mentioned before, some BceRS-like systems have a bigger regulon controlling additional genes. Usually these additional genes include those mediating the cell wall stress response, like *dltABCD* and *mprF*. This is the case of Aps/GraRS from *S. aureus* (Li *et al.*, 2007), VirRS from *L. monocytogenes* (Mandin *et al.*, 2005; Camejo *et al.*, 2009) and ApsRS of *S. epidermidis* (Li *et al.*, 2007). Also, genes for cell wall biosynthesis can be found between the Bce-like systems target genes (Kolar *et al.*, 2011; Falord *et al.*, 2012). Expression of these set of genes controlling properties of the cell surface would undoubtedly help to counteract the AMP damaging effects.

7 TCS in *Lactobacillus casei* BL23.

Different studies have addressed the role of TCS in the biology and stress response in *Lactobacillus acidophilus* (Azcarate-Peril *et al.*, 2005; Pfeiler *et al.*, 2007; Cui and Qu, 2011), *Lactobacillus plantarum* (Risoen *et al.*, 1998; Sturme *et al.*, 2005; Fujii *et al.*, 2008; Maldonado-Barragán *et al.*, 2009), *Lactobacillus sakei* (Morel-Deville *et al.*, 1998) and *Lactobacillus delbrueckii* (Cui *et al.*, 2012). However, no studies on TCS in *Lactobacillus casei* had been reported when this PhD thesis started.

The availability of the complete genome sequence of *L. casei* strain BL23 (Mazé *et al.*, 2010) enabled a comprehensive study of the TCS present in this organism. Inspection of its genome sequence revealed the presence of 17 putative TCS all of them consisting of cognate HK and RR pairs excepting TCS13 where the HK may not be functional due to a possible frameshift in its coding sequence (Figure 13) (Alcántara *et al.*, 2011). However, this HK is intact in other *L. casei* strains (Nakayama *et al.*, 2003). They were renamed numerically from TCS01 to TCS17 (Figure 13) (Alcántara *et al.*, 2011). TCS17 correspond to the previously characterized system MaeKR (Landete *et al.*, 2010). The analysis of conserved domains predicted that all HKs are anchored to the membrane.

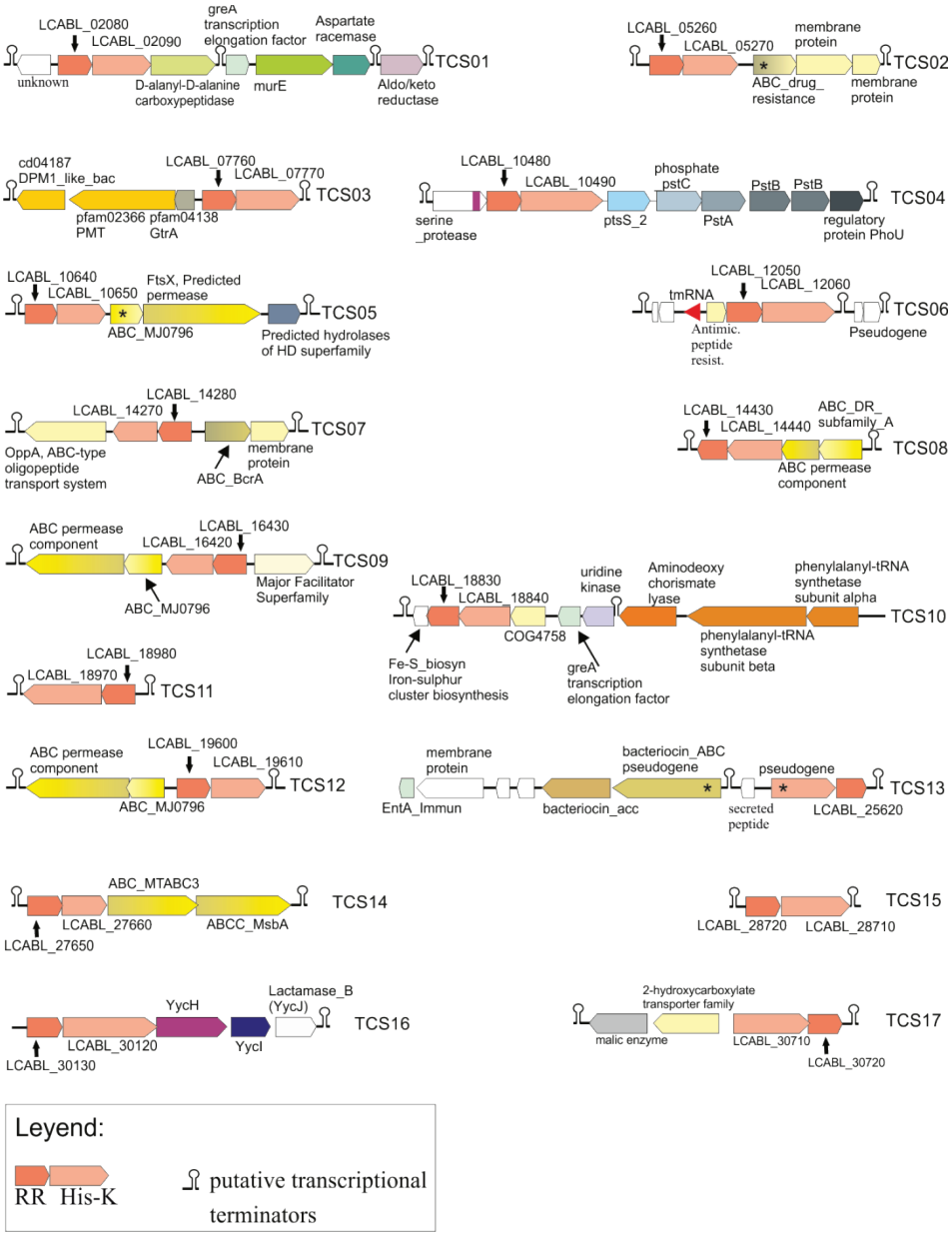


Figure 13 . Schematic representation of clusters containing genes encoding Two Component Systems in *Lactobacillus casei* BL23. Filling patterns indicate homologous genes. Asterisks indicate putative pseudogenes.

The inspection of the genomic context of *L. casei* TCS showed that most of them were located in clusters with other genes (Figure 13). Besides the aforementioned TCS17 involved in L-malic acid metabolism (Landete *et al.*, 2010), TCS01 is clustered with proteins involved in cell wall synthesis, TCS03 with proteins putatively involved in glycosylation of cell envelope components and TCS04 with genes encoding a putative phosphate transporter. Only TCS11 and TCS15 constituted simple operons, whereas seven systems were clustered with genes encoding putative ABC transporters. At least two TCS included auxiliary proteins: TCS10 is homologous to the *Bacillus subtilis* LiaRS system and TCS16 to the YycFG system. In *B. subtilis*, the auxiliary protein LiaF is a strong inhibitor of LiaR-dependent gene expression (Jordan *et al.*, 2006); an homolog (LCABL_18850, COG4758) is also present in *L. casei* (Figure 13). The system *yycFG* constitute and operon with genes *yycH*, *yycI* and *yycJ* in *B. subtilis*. YycH and YycI repress the activity of the HK YycG (Winkler and Hoch, 2008). Finally, TCS06 and TCS13 are clustered with putative uncharacterized proteins. The TCS06 cluster also includes a putative gene encoding a transfer-messenger RNA (Figure 13).

In this work, we deepen in the involvement of TCS in the biology and stress response of *L. casei* BL23. These results will be described in Chapter 1 (Alcántara *et al.*, 2011). We further study the involvement of the Bce-like TCS of *L. casei* BL23 (TCS09 and TCS12) in AMP resistance (results presented in Chapter 2 (Revilla-Guarinos *et al.*, 2013)) and in acid stress response (TCS12; results presented in Chapter 3 (Revilla-Guarinos *et al.*, 2014)).

REFERENCES

Abachin, E., C. Poyart*, et al.* (2002). "Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*." Mol Microbiol **43**(1): 1-14.

Abdelhady, W., A. S. Bayer*, et al.* (2013). "Reduced vancomycin susceptibility in an in vitro catheter-related biofilm model correlates with poor therapeutic outcomes in experimental endocarditis due to methicillin-resistant *Staphylococcus aureus*." Antimicrob Agents Chemother **57**(3): 1447-1454.

Acedo-Félix, E. and G. Pérez-Martínez (2003). "Significant differences between *Lactobacillus casei subsp. casei* ATCC 393T and a commonly used plasmid-cured derivative revealed by a polyphasic study." Int J Syst Evol Microbiol **53**(1): 67-75.

Adamson, A. W. (1982). Electrical aspects of surface chemistry. . Physical Chemistry of Surfaces. N. Y. John Wiley and Sons**:** 185–229.

Alcántara, C., A. Revilla-Guarinos*, et al.* (2011). "Influence of Two-Component Signal Transduction Systems of *Lactobacillus casei* BL23 on Tolerance to Stress Conditions." Appl. Environ. Microbiol. **77**(4): 1516-1519.

Alcántara, C. and M. Zúñiga (2012). "Proteomic and transcriptomic analysis of the response to bile stress of *Lactobacillus casei* BL23." Microbiology **158**(Pt 5): 1206-1218.

Anaya-López, J. L., J. E. López-Meza*, et al.* (2013). "Bacterial resistance to cationic antimicrobial peptides." Crit. Rev. Microbiol. **39**(2): 180-195.

Arthur, R. A., R. A. Waeiss*, et al.* (2013). "A defined-multispecies microbial model for studying enamel caries development." Caries Res **47**(4): 318-324.

Azcarate-Peril, M. A., O. McAuliffe*, et al.* (2005). "Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in Lactobacillus acidophilus." Appl. Environ. Microbiol. **71**(10): 5794-5804.

Aznar, R. and M. Zúñiga. (2011). "¿Qué son las bacterias lácticas?", from http://redbal.iata.csic.es/sabias_que.php.

Barakat, M., P. Ortet*, et al.* (2011). "P2CS: a database of prokaryotic two-component systems." Nucleic Acids Res **39**(suppl 1): D771-D776.

Barreteau, H., S. Magnet*, et al.* (2009). "Quantitative high-performance liquid chromatography analysis of the pool levels of undecaprenyl phosphate and its derivatives in bacterial membranes." J Chromatogr B Analyt Technol Biomed Life Sci **877**(3): 213-220.

Bäuerl, C., M. Llopis*, et al.* (2013). "*Lactobacillus paracasei* and *Lactobacillus plantarum* strains downregulate proinflammatory genes in an ex vivo system of cultured human colonic mucosa." Genes & Nutrition **8**(2): 165-180.

Beeby, M., J. C. Gumbart*, et al.* (2013). "Architecture and assembly of the Gram-positive cell wall." Mol Microbiol **88**(4): 664-672.

Benoit Foligne, S. N., Corinne Grangette, Véronique Dennin, Denise Goudercourt, Sabine Poiret, and D. B. Joelle Dewulf, Annick Mercenier, Bruno Pot (2007). "Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria." World Journal of Gastroenterology **13**: 236-243.

Benson, A. K., S. A. Kelly*, et al.* (2010). "Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors." Proceedings of the National Academy of Sciences **107**(44): 18933-18938.

Berditsch, M., S. Afonin*, et al.* (2012). "Antimicrobial Peptides can Enhance the Risk of Persistent Infections." Front Immunol **3**: 222.

Bernard, R., M. El Ghachi*, et al.* (2005). "BcrC from *Bacillus subtilis* acts as an undecaprenyl pyrophosphate phosphatase in bacitracin resistance." J. Biol. Chem. **280**(32): 28852-28857.

Bernard, R., A. Guiseppi*, et al.* (2007). "Resistance to bacitracin in *Bacillus subtilis*: unexpected requirement of the BceAB ABC transporter in the control of expression of its own structural genes." J Bacteriol **189**(23): 8636-8642.

Biavati, B. and P. Mattarelli (2006). The Family Bifidobacteriaceae. The Prokaryotes. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt, Springer New York**:** 322-382.

Bierbaum, G. and H. G. Sahl (2009). "Lantibiotics: mode of action, biosynthesis and bioengineering." Curr Pharm Biotechnol **10**(1): 2-18.

Biswas, I., L. Drake*, et al.* (2008). "Involvement of sensor kinases in the stress tolerance response of *Streptococcus mutans*." J Bacteriol **190**(1): 68-77.

Blake, R. C., E. A. Shute*, et al.* (1994). "Solubilization of Minerals by Bacteria: Electrophoretic Mobility of *Thiobacillus ferrooxidans* in the Presence of Iron, Pyrite, and Sulfur." Appl Environ Microbiol **60**(9): 3349-3357.

Bolhuis, H., H. W. van Veen*, et al.* (1996). "Multidrug resistance in *Lactococcus lactis:* evidence for ATP-dependent drug extrusion from the inner leaflet of the cytoplasmic membrane." EMBO J **15**(16): 4239-4245.

Bonev, B. B., E. Breukink*, et al.* (2004). "Targeting extracellular pyrophosphates underpins the high selectivity of nisin." FASEB J **18**(15): 1862-1869.

Booijink, C. C., S. El-Aidy*, et al.* (2010). "High temporal and inter-individual variation detected in the human ileal microbiota." Environ Microbiol **12**(12): 3213-3227.

Boonaert, C. J. P. and P. G. Rouxhet (2000). "Surface of Lactic Acid Bacteria: Relationships between Chemical Composition and Physicochemical Properties." Appl Environ Microbiol **66**(6): 2548-2554.

Bouhss, A., A. E. Trunkfield*, et al.* (2008). "The biosynthesis of peptidoglycan lipid-linked intermediates." FEMS Microbiol Rev **32**(2): 208-233.

Bourand, A., M. J. Yebra*, et al.* (2013). "Utilization of d-Ribitol by *Lactobacillus casei* BL23 Requires a Mannose-Type Phosphotransferase System and Three Catabolic Enzymes." J Bacteriol **195**(11): 2652-2661.

Bourlioux, P., B. Koletzko*, et al.* (2003). "The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium "The Intelligent Intestine," held in Paris, June 14, 2002." The American Journal of Clinical Nutrition **78**(4): 675-683.

Boyd, D. A., D. G. Cvitkovitch*, et al.* (2000). "Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity." J. Bacteriol. **182**(21): 6055-6065.

Breukink, E., I. Wiedemann*, et al.* (1999). "Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic." Science **286**(5448): 2361-2364.

Brinton, J. C. C., Lauffer, M.A. (1959). The electrophoresis of viruses, bacteria, and cells, and the microscope method of electrophoresis. Electrophoresis Theory, Methods, and Applications. M. Bier, Academic Press Inc, New York**:** 427–492.

Broadbent, J. R., R. L. Larsen*, et al.* (2010). "Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress." J. Bacteriol. **192**(9): 2445-2458.

Bron, P. A. and M. Kleerebezem (2011). "Engineering lactic acid bacteria for increased industrial functionality." Bioeng Bugs **2**(2): 80-87.

Brötz, H., M. Josten*, et al.* (1998). "Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics." Mol. Microbiol. **30**(2): 317-327.

Buchanan, R. L. and S. G. Edelson (1996). "Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells." Appl Environ Microbiol **62**(11): 4009-4013.

Cai, H., R. Thompson*, et al.* (2009). "Genome sequence and comparative genome analysis of *Lactobacillus casei*: insights into their niche-associated evolution." Genome Biol Evol **1**: 239-257.

Camejo, A., C. Buchrieser*, et al.* (2009). "In vivo transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection." PLoS Pathog **5**(5): e1000449.

Cao, M. and J. D. Helmann (2002). "Regulation of the *Bacillus subtilis bcrC* bacitracin resistance gene by two extracytoplasmic function sigma factors." J Bacteriol **184**(22): 6123-6129.

Cao, M. and J. D. Helmann (2004). "The *Bacillus subtilis* extracytoplasmic-function σ^X factor regulates modification of the cell envelope and resistance to cationic antimicrobial peptides." J Bacteriol **186**(4): 1136-1146.

Capra, E. J. and M. T. Laub (2012). "Evolution of two-component signal transduction systems." Annu Rev Microbiol **66**: 325-347.

Clarke, G., J. F. Cryan*, et al.* (2012). "Review article: probiotics for the treatment of irritable bowel syndrome--focus on lactic acid bacteria." Aliment Pharmacol Ther **35**(4): 403-413.

Collins, B., N. Curtis*, et al.* (2010). "The ABC transporter AnrAB contributes to the innate resistance of *Listeria monocytogenes* to nisin, bacitracin, and various beta-lactam antibiotics." Antimicrob. Agents Chemother. **54**(10): 4416-4423.

Collins, M. D., B. A. Phillips*, et al.* (1989). "Deoxyribonucleic Acid Homology Studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov." International Journal of Systematic Bacteriology **39**(2): 105-108.

Corcoran, B. M., C. Stanton*, et al.* (2008). "Life under stress: the probiotic stress response and how it may be manipulated." Curr. Pharm. Des. **14**(14): 1382-1399.

Cortese, J. D., A. L. Voglino*, et al.* (1995). "Persistence of cytochrome *c* binding to membranes at physiological mitochondrial intermembrane space ionic strength." Biochim Biophys Acta **1228**(2–3): 216-228.

Cotter, P. D. and C. Hill (2003). "Surviving the acid test: responses of gram-positive bacteria to low pH." Microbiol. Mol. Biol. Rev. **67**(3): 429-453, table of contents.

Cotter, P. D., C. Hill*, et al.* (2005). "Bacteriocins: developing innate immunity for food." Nat. Rev. Microbiol. **3**(10): 777-788.

Cui, Y., W. Liu*, et al.* (2012). "A two component system is involved in acid adaptation of *Lactobacillus delbrueckii* subsp. *bulgaricus*." Microbiological Research **167**(5): 253-261.

Cui, Y. and X. Qu (2011). "Comparative Analysis of two Component Signal Transduction Systems of the *Lactobacillus Acidophilus* Group." Braz J Microbiol **42**(1): 147-157.

Cunin, R., N. Glansdorff*, et al.* (1986). "Biosynthesis and metabolism of arginine in bacteria." Microbiol Rev **50**(3): 314-352.

Cunliffe, R. N. (2003). "Alpha-defensins in the gastrointestinal tract." Mol. Immunol. **40**(7): 463-467.

Chang, G. (2003). "Multidrug resistance ABC transporters." FEBS Letters **555**(1): 102-105.

Chang, Y. Y. and J. E. Cronan, Jr. (1999). "Membrane cyclopropane fatty acid content is a major factor in acid resistance of Escherichia coli." Mol Microbiol **33**(2): 249-259.

Davie, J. M. and T. D. Brock (1966). "Effect of teichoic acid on resistance to membrane-lytic agent of *Streptococcus zymogenes*." J. Bacteriol. **92**(6): 1623-&.

De Angelis, M. and M. Gobbetti (2004). "Environmental stress responses in *Lactobacillus*: a review." PROTEOMICS **4**(1): 106-122.

de Been, M., M. J. Bart*, et al.* (2008). "The identification of response regulator-specific binding sites reveals new roles of two-component systems in *Bacillus cereus*

and closely related low-GC Gram-positives." Environ Microbiol **10**(10): 2796-2809.

de Jong, A., S. A. van Hijum*, et al.* (2006). "BAGEL: a web-based bacteriocin genome mining tool." Nucleic Acids Res **34**(Web Server issue): W273-279.

de Vrese, M. and J. Schrezenmeir (2008). Probiotics, prebiotics, and synbiotics. Adv. Biochem. Eng. Biotechnol. **111:** 1-66.

Deepika, G. and D. Charalampopoulos (2010). "Surface and adhesion properties of lactobacilli." Adv Appl Microbiol **70**: 127-152.

Deepika, G., R. J. Green*, et al.* (2009). "Effect of growth time on the surface and adhesion properties of *Lactobacillus rhamnosus* GG." J Appl Microbiol **107**(4): 1230-1240.

Delcour, J., T. Ferain*, et al.* (1999). "The biosynthesis and functionality of the cell-wall of lactic acid bacteria." Antonie Van Leeuwenhoek **76**(1-4): 159-184.

Dellaglio, F., G. E. Felis*, et al.* (2002). "The status of the species *Lactobacillus casei* (Orla-Jensen 1916) Hansen and Lessel 1971 and *Lactobacillus paracasei* Collins et al. 1989. Request for an opinion." Int J Syst Evol Microbiol **52**(1): 285-287.

Dintner, S., A. Staroń*, et al.* (2011). "Coevolution of ABC transporters and two-component regulatory systems as resistance modules against antimicrobial peptides in *Firmicutes* Bacteria." J. Bacteriol. **193**(15): 3851-3862.

Dommett, R., M. Zilbauer*, et al.* (2005). "Innate immune defence in the human gastrointestinal tract." Mol. Immunol. **42**(8): 903-912.

Doyle, R. J., M. L. McDannel*, et al.* (1974). "Polyelectrolyte nature of bacterial teichoic acids." J Bacteriol **118**(2): 606-615.

Dunman, P. M., E. Murphy*, et al.* (2001). "Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci." J Bacteriol **183**(24): 7341-7353.

Eckburg, P. B., E. M. Bik*, et al.* (2005). "Diversity of the human intestinal microbial flora." Science **308**(5728): 1635-1638.

Economou, N. J., S. Cocklin*, et al.* (2013). "High-resolution crystal structure reveals molecular details of target recognition by bacitracin." Proc Natl Acad Sci U S A **110**(35): 14207-14212.

Eitinger, T., D. A. Rodionov*, et al.* (2011). "Canonical and ECF-type ATP-binding cassette importers in prokaryotes: diversity in modular organization and cellular functions." FEMS Microbiology Reviews **35**(1): 3-67.

Ellwood, D. C. and D. W. Tempest (1972). "Influence of culture pH on the content and composition of teichoic acids in the walls of *Bacillus subtilis*." J Gen Microbiol **73**(2): 395-402.

Ernst, C. M. and A. Peschel (2011). "Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids." Mol. Microbiol. **80**(2): 290-299.

Ernst, C. M., P. Staubitz*, et al.* (2009). "The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion." PLoS Pathog. **5**(11): e1000660.

European Economic Community, E. (1983). E. E. C. C. Directive. Official J. European Union, 255. **83/463/EEC:** 1-6.

Fabret, C., V. A. Feher*, et al.* (1999). "Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world." J Bacteriol **181**(7): 1975-1983.

Fabretti, F., C. Theilacker*, et al.* (2006). "Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides." Infect Immun **74**(7): 4164-4171.

Falord, M., G. Karimova*, et al.* (2012). "GraXSR proteins interact with the VraFG ABC transporter to form a five-component system required for cationic antimicrobial peptide sensing and resistance in *Staphylococcus aureus*." Antimicrob. Agents Chemother. **56**(2): 1047-1058.

Falord, M., U. Mäder*, et al.* (2011). "Investigation of the *Staphylococcus aureus* GraSR regulon reveals novel links to virulence, stress response and cell wall signal transduction pathways." PLoS ONE **6**(7): e21323.

Fang, X., K. Tiyanont*, et al.* (2006). "The mechanism of action of ramoplanin and enduracidin." Mol Biosyst **2**(1): 69-76.

Fernandes, C. F., R. C. Chandan*, et al.* (1992). Fermented Dairy Products and Health. The Lactic Acid Bacteria Volume 1. B. B. Wood, Springer US**:** 297-339.

Fernandez, A., J. Ogawa*, et al.* (2008). "Rerouting of pyruvate metabolism during acid adaptation in *Lactobacillus bulgaricus*." PROTEOMICS **8**(15): 3154-3163.

Food and Agriculture Organization of the United Nations & World Health Organization, F. W. (2006). "Probiotics in food: health and nutritional properties and guidelines for evaluation." Food and Agriculture Organization of the United Nations: World Health Organization, Rome. .

Fozo, E. M., J. K. Kajfasz*, et al.* (2004). "Low pH-induced membrane fatty acid alterations in oral bacteria." FEMS Microbiol Lett **238**(2): 291-295.

Fujii, T., C. Ingham*, et al.* (2008). "Two homologous Agr-like quorum-sensing systems cooperatively control adherence, cell morphology, and cell viability properties in *Lactobacillus plantarum* WCFS1." J Bacteriol **190**(23): 7655-7665.

Gálvez, A., H. Abriouel*, et al.* (2007). "Bacteriocin-based strategies for food biopreservation." Int. J. Food Microbiol. **120**(1-2): 51-70.

Gao, R. and A. M. Stock (2009). "Biological insights from structures of two-component proteins." Annu. Rev. Microbiol. **63**: 133-154.

García, M. J., M. Zúñiga*, et al.* (1992). "Energy production from L-malic acid degradation and protection against acidic external pH in *Lactobacillus plantarum* CECT 220." J. Gen. Microbiol. **138**(12): 2519-2524.

Gebhard, S. (2012). "ABC transporters of antimicrobial peptides in Firmicutes bacteria - phylogeny, function and regulation." Mol Microbiol **86**(6): 1295-1317.

Gebhard, S. and T. Mascher (2011). "Antimicrobial peptide sensing and detoxification modules: unravelling the regulatory circuitry of *Staphylococcus aureus*." Mol. Microbiol. **81**(3): 581-587.

Ghuysen, J. M. (1991). "Serine beta-lactamases and penicillin-binding proteins." Annu Rev Microbiol **45**: 37-67.

Giaouris, E., R. Briandet*, et al.* (2008). "Variations in the degree of D-Alanylation of teichoic acids in *Lactococcus lactis* alter resistance to cationic antimicrobials but have no effect on bacterial surface hydrophobicity and charge." Appl Environ Microbiol **74**(15): 4764-4767.

Gionchetti, P., F. Rizzello*, et al.* (2000). "Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial." Gastroenterology **119**(2): 305-309.

Gomes, D. L., R. S. Peixoto*, et al.* (2013). "SubMICs of penicillin and erythromycin enhance biofilm formation and hydrophobicity of *Corynebacterium diphtheriae* strains." J Med Microbiol **62**(Pt 5): 754-760.

Goodson, M. and R. J. Rowbury (1989). "Habituation to normally lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value." Lett Appl Microbiol **8**(2): 77-79.

Gordon, S. (2008). "Elie Metchnikoff: Father of natural immunity." European Journal of Immunology **38**(12): 3257-3264.

Grebe, T. W. and J. B. Stock (1999). "The histidine protein kinase superfamily." Adv Microb Physiol **41**: 139-227.

Guariglia-Oropeza, V. and J. D. Helmann (2011). "*Bacillus subtilis* σ^V confers lysozyme resistance by activation of two cell wall modification pathways, peptidoglycan O-acetylation and D-alanylation of teichoic acids." J Bacteriol **193**(22): 6223-6232.

Hall-Stoodley, L., J. W. Costerton*, et al.* (2004). "Bacterial biofilms: from the Natural environment to infectious diseases." Nat Rev Micro **2**(2): 95-108.

Haller, D., C. Bode*, et al.* (2000). "Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures." Gut **47**(1): 79-87.

Hamilton, I. R. and G. Svensäter (1998). "Acid-regulated proteins induced by *Streptococcus mutans* and other oral bacteria during acid shock." Oral Microbiol. Immunol. **13**(5): 292-300.

Hammes, W. P. and C. Hertel (2006). The Genera *Lactobacillus* and *Carnobacterium*. The Prokaryotes. M. D. P. Dr., S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt, Springer US. **Vol. 4**.

Hancock, L. E. and M. Perego (2004). "Systematic inactivation and phenotypic characterization of two-component signal transduction systems of *Enterococcus faecalis* V583." J. Bacteriol. **186**(23): 7951-7958.

Hancock, R. E. and G. Diamond (2000). "The role of cationic antimicrobial peptides in innate host defences." Trends Microbiol. **8**(9): 402-410.

Hancock, R. E. W. and D. S. Chapple (1999). "Peptide Antibiotics." Antimicrobial Agents and Chemotherapy **43**(6): 1317-1323.

Havarstein, L. S., D. B. Diep*, et al.* (1995). "A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export." Mol Microbiol **16**(2): 229-240.

Hayashi, H., R. Takahashi*, et al.* (2005). "Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism." J Med Microbiol **54**(Pt 11): 1093-1101.

Hazen, T. C. and D. A. Stahl (2006). "Using the stress response to monitor process control: pathways to more effective bioremediation." Curr Opin Biotechnol **17**(3): 285-290.

Heimann, J. D. (2002). The extracytoplasmic function (ECF) sigma factors. Adv Microb Physiol, Academic Press. **Volume 46:** 47-110.

Heptinstall, S., A. R. Archibald*, et al.* (1970). "Teichoic acids and membrane function in bacteria." Nature **225**(5232): 519-521.

Hirayama, K. and J. Rafter (1999). "The role of lactic acid bacteria in colon cancer prevention: mechanistic considerations." Antonie Van Leeuwenhoek **76**(1-4): 391-394.

Hiron, A., M. Falord*, et al.* (2011). "Bacitracin and nisin resistance in *Staphylococcus aureus*: a novel pathway involving the BraS/BraR two-component system (SA2417/SA2418) and both the BraD/BraE and VraD/VraE ABC transporters." Mol. Microbiol. **81**(3): 602-622.

Hollenstein, K., R. J. Dawson*, et al.* (2007). "Structure and mechanism of ABC transporter proteins." Curr Opin Struct Biol **17**(4): 412-418.

Hong, Y. and D. G. Brown (2008). "Electrostatic Behavior of the Charge-Regulated Bacterial Cell Surface." Langmuir **24**(9): 5003-5009.

Howden, C. W. and R. H. Hunt (1987). "Relationship between gastric secretion and infection." Gut **28**(1): 96-107.

Hsu, S. T., E. Breukink*, et al.* (2004). "The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics." Nat Struct Mol Biol **11**(10): 963-967.

Hunter, R. J. (1993). Electrified interfaces: the electrical double layer. Foundations of Colloid Science. N. Y. Oxford Univesity Press. **1:** 316–394.

Husebye, E. (2005). "The pathogenesis of gastrointestinal bacterial overgrowth." Chemotherapy **51 Suppl 1**: 1-22.

Hutkins, R. W. a. N., Nancy L. (1993). "pH Homeostasis in Lactic Acid Bacteria." University of Nebraska-Lincoln, Faculty Publications in Food Science and Technology **76**(J Dairy Sci): 2354-2365.

Huynh, T. N. and V. Stewart (2011). "Negative control in two-component signal transduction by transmitter phosphatase activity." Mol Microbiol **82**(2): 275-286.

Jiménez, E., M. L. Marín*, et al.* (2008). "Is meconium from healthy newborns actually sterile?" Research in Microbiology **159**(3): 187-193.

Johnson, B. A., H. Anker*, et al.* (1945). "Bacitracin: A new antibiotic produced by a member of the *B. subtilis* group." Science **102**(2650): 376-377.

Jones, P. M. and A. M. George (2004). "The ABC transporter structure and mechanism: perspectives on recent research." Cell Mol Life Sci **61**(6): 682-699.

Jordan, S., M. I. Hutchings*, et al.* (2008). "Cell envelope stress response in Gram-positive bacteria." FEMS Microbiology Reviews **32**(1): 107-146.

Jordan, S., A. Junker*, et al.* (2006). "Regulation of LiaRS-dependent gene expression in *bacillus subtilis*: identification of inhibitor proteins, regulator binding sites, and target genes of a conserved cell envelope stress-sensing two-component system." J Bacteriol **188**(14): 5153-5166.

Joseph, P., A. Guiseppi*, et al.* (2004). "Characterization of the *Bacillus subtilis* YxdJ response regulator as the inducer of expression for the cognate ABC transporter YxdLM." Microbiology **150**(Pt 8): 2609-2617.

Jung, K., L. Fried*, et al.* (2012). "Histidine kinases and response regulators in networks." Curr Opin Microbiol **15**(2): 118-124.

Kallenberg, F., S. Dintner*, et al.* (2013). "Identification of regions important for resistance and signalling within the antimicrobial peptide transporter BceAB of *Bacillus subtilis*." J Bacteriol **195**(14): 3287-3297.

Kankainen, M., L. Paulin*, et al.* (2009). "Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human- mucus binding protein." Proc Natl Acad Sci U S A **106**(40): 17193-17198.

Kanmani, P., R. Satish Kumar*, et al.* (2012). "Probiotics and Its Functionally Valuable Products—A Review." Critical Reviews in Food Science and Nutrition **53**(6): 641-658.

Kanno, T., T. Matsuki*, et al.* (2009). "Gastric acid reduction leads to an alteration in lower intestinal microflora." Biochem Biophys Res Commun **381**(4): 666-670.

Kaplan, J. B. (2011). "Antibiotic-induced biofilm formation." Int J Artif Organs **34**(9): 737-751.

Kaplan, J. B., E. A. Izano*, et al.* (2012). "Low levels of beta-lactam antibiotics induce extracellular DNA release and biofilm formation in *Staphylococcus aureus*." MBio **3**(4): e00198-00112.

Kashket, E. R. (1987). "Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance." FEMS Microbiology Letters **46**(3): 233-244.

Kawada-Matsuo, M., Y. Yoshida*, et al.* (2011). "Role of two-component systems in the resistance of *Staphylococcus aureus* to antibacterial agents." Virulence **2**(5): 427-430.

Kingston, A. W., X. Liao*, et al.* (2013). "Contributions of the σW, σM, and σX regulons to the lantibiotic resistome of *Bacillus subtilis*." Mol Microbiol: DOI: 10.1111/mmi.12380.

Klaenhammer, T. R. (1993). "Genetics of bacteriocins produced by lactic acid bacteria." FEMS Microbiol. Rev. **12**(1-3): 39-85.

Kleerebezem, M., P. Hols*, et al.* (2010). "The extracellular biology of the lactobacilli." FEMS Microbiology Reviews **34**(2): 199-230.

Kleerebezem, M. and E. E. Vaughan (2009). "Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity." Annu. Rev. Microbiol. **63**: 269-290.

Kłodzińska, E., M. Szumski*, et al.* (2010). "Effect of zeta potential value on bacterial behavior during electrophoretic separation." Electrophoresis **31**(9): 1590-1596.

Koenig, J. E., A. Spor*, et al.* (2011). "Succession of microbial consortia in the developing infant gut microbiome." Proceedings of the National Academy of Sciences **108**(Supplement 1): 4578-4585.

Kolar, S. L., V. Nagarajan*, et al.* (2011). "NsaRS is a cell-envelope-stress-sensing two-component system of *Staphylococcus aureus*." Microbiology **157**(Pt 8): 2206-2219.

Koponen, J., K. Laakso*, et al.* (2012). "Effect of acid stress on protein expression and phosphorylation in *Lactobacillus rhamnosus* GG." J Proteomics **75**(4): 1357-1374.

Koponen, O., T. M. Takala*, et al.* (2004). "Distribution of the NisI immunity protein and enhancement of nisin activity by the lipid-free NisI." FEMS Microbiol Lett **231**(1): 85-90.

Koprivnjak, T., V. Mlakar*, et al.* (2006). "Cation-induced transcriptional regulation of the dlt operon of Staphylococcus aureus." J Bacteriol **188**(10): 3622-3630.

Koretke, K. K., A. N. Lupas*, et al.* (2000). "Evolution of two-component signal transduction." Mol Biol Evol **17**(12): 1956-1970.

Kovács, M., A. Halfmann*, et al.* (2006). "A functional *dlt* operon, encoding proteins required for incorporation of D-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in *Streptococcus pneumoniae*." J Bacteriol **188**(16): 5797-5805.

Kristian, S. A., V. Datta*, et al.* (2005). "D-alanylation of teichoic acids promotes group A *Streptococcus* antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion." J Bacteriol **187**(19): 6719-6725.

Lambert, R. J. and M. Stratford (1999). "Weak-acid preservatives: modelling microbial inhibition and response." J Appl Microbiol **86**(1): 157-164.

Landete, J. M., S. Ferrer*, et al.* (2013). "Malic Enzyme and Malolactic Enzyme Pathways Are Functionally Linked but Independently Regulated in *Lactobacillus casei* BL23." Appl Environ Microbiol **79**(18): 5509-5518.

Landete, J. M., L. García-Haro*, et al.* (2010). "Requirement of the *Lactobacillus casei* MaeKR two-component system for L-malic acid utilization via a malic enzyme pathway." Appl. Environ. Microbiol. **76**(1): 84-95.

Ley, R. E., D. A. Peterson*, et al.* (2006). "Ecological and Evolutionary Forces Shaping Microbial Diversity in the Human Intestine." Cell **124**(4): 837-848.

Li, H., M. Lu*, et al.* (2010). "Protective Effect of Sucrose on the Membrane Properties of *Lactobacillus case*i Zhang Subjected to Freeze-Drying." Journal of Food Protection **73**(4): 715-719.

Li, M., D. J. Cha*, et al.* (2007). "The antimicrobial peptide-sensing system aps of *Staphylococcus aureus*." Mol. Microbiol. **66**(5): 1136-1147.

Li, M., Y. Lai*, et al.* (2007). "Gram-positive three-component antimicrobial peptide-sensing system." Proc. Natl. Acad. Sci. U.S.A. **104**(22): 9469-9474.

Liu, Z., W. Wang*, et al.* (2013). "Antibiotics at subinhibitory concentrations improve the quorum sensing behavior of *Chromobacterium violaceum*." FEMS Microbiology Letters **341**(1): 37-44.

Locher, K. P. (2004). "Structure and mechanism of ABC transporters." Curr Opin Struct Biol **14**(4): 426-431.

Madigan, M. T., J. M. Martinko*, et al.* (2012). Brock, Biology of Microorganisms.

Madsen, K. L., J. S. Doyle*, et al.* (1999). "Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice." Gastroenterology **116**(5): 1107-1114.

Mah, T.-F. C. and G. A. O'Toole (2001). "Mechanisms of biofilm resistance to antimicrobial agents." Trends Microbiol **9**(1): 34-39.

Mahowald, M. A., F. E. Rey*, et al.* (2009). "Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla." Proc Natl Acad Sci U S A **106**(14): 5859-5864.

Makinen, K., B. Berger*, et al.* (2012). "Science and technology for the mastership of probiotic applications in food products." Journal of Biotechnology **162**(4): 356-365.

Maldonado-Barragán, A., J. L. Ruiz-Barba*, et al.* (2009). "Knockout of three-component regulatory systems reveals that the apparently constitutive plantaricin-production phenotype shown by *Lactobacillus plantarum* on solid medium is regulated via quorum sensing." Int J Food Microbiol **130**(1): 35-42.

Mandin, P., H. Fsihi*, et al.* (2005). "VirR, a response regulator critical for *Listeria monocytogenes* virulence." Mol. Microbiol. **57**(5): 1367-1380.

Marchesi, J. R. (2010). Chapter 2 - Prokaryotic and Eukaryotic Diversity of the Human Gut. Advances in Applied Microbiology. S. S. Allen I. Laskin and M. G. Geoffrey, Academic Press. **Volume 72:** 43-62.

Martin, R., S. Miquel*, et al.* (2013). "Role of commensal and probiotic bacteria in human health: a focus on inflammatory bowel disease." Microb Cell Fact **12**: 71.

Mascher, T. (2006). "Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in Firmicutes bacteria." FEMS Microbiology Letters **264**(2): 133-144.

Mascher, T., M. Heintz*, et al.* (2006). "The CiaRH system of *Streptococcus pneumoniae* prevents lysis during stress induced by treatment with cell wall inhibitors and by mutations in pbp2x involved in beta-lactam resistance." J Bacteriol **188**(5): 1959-1968.

Mascher, T., J. D. Helmann*, et al.* (2006). "Stimulus Perception in Bacterial Signal-Transducing Histidine Kinases." Microbiology and Molecular Biology Reviews **70**(4): 910-938.

Mascher, T., N. G. Margulis*, et al.* (2003). "Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon." Mol. Microbiol. **50**(5): 1591-1604.

Matias, V. R. and T. J. Beveridge (2005). "Cryo-electron microscopy reveals native polymeric cell wall structure in *Bacillus subtilis* 168 and the existence of a periplasmic space." Mol Microbiol **56**(1): 240-251.

Matsuo, M., Y. Oogai*, et al.* (2011). "Growth-phase dependence of susceptibility to antimicrobial peptides in *Staphylococcus aureus*." Microbiology **157**(Pt 6): 1786-1797.

Mazé, A., G. Boël*, et al.* (2010). "Complete Genome Sequence of the Probiotic *Lactobacillus casei* Strain BL23." J Bacteriol **192**(10): 2647-2648.

McCormick, N. E., S. A. Halperin*, et al.* (2011). "Regulation of D-alanylation of lipoteichoic acid in *Streptococcus gordonii*." Microbiology **157**: 2248-2256.

Meehl, M., S. Herbert*, et al.* (2007). "Interaction of the GraRS two-component system with the VraFG ABC transporter to support vancomycin-intermediate resistance in *Staphylococcus aureus*." Antimicrob. Agents Chemother. **51**(8): 2679-2689.

Millsap, K. W., G. Reid*, et al.* (1997). "Cluster analysis of genotypically characterized *Lactobacillus* species based on physicochemical cell surface properties and their relationship with adhesion to hexadecane." Can J Microbiol **43**(3): 284-291.

Ming, L.-J. and J. D. Epperson (2002). "Metal binding and structure–activity relationship of the metalloantibiotic peptide bacitracin." J. Inorg. Biochem. **91**(1): 46-58.

Modesto, A., K. C. Lima*, et al.* (2000). "Effects of three different infant dentifrices on biofilms and oral microorganisms." J Clin Pediatr Dent **24**(3): 237-243.

Mohammadi, T., V. van Dam*, et al.* (2011). "Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane." EMBO J **30**(8): 1425-1432.

Morel-Deville, F., F. Fauvel*, et al.* (1998). "Two-component signal-transducing systems involved in stress responses and vancomycin susceptibility in *Lactobacillus sakei*." Microbiology **144 (Pt 10)**: 2873-2883.

Moyer, L. S. (1936). "A Suggested Standard Method for the Investigation of Electrophoresis." J Bacteriol **31**(5): 531-546.

Mozes, N., Rouxhet, P.G. (1990). Microbial hydrophobicity and fermentation tehcnology. Microbial Cell Surface Hydrophobicity. R. J. Doyle, Rosenberg, M. , American Society for Microbiology, Washington, DC, USA.**:** 75-105.

Muñoz-Provencio, D., M. Llopis*, et al.* (2009). "Adhesion properties of *Lactobacillus casei* strains to resected intestinal fragments and components of the extracellular matrix." Arch Microbiol **191**(2): 153-161.

Muñoz-Provencio, D., G. Pérez-Martínez*, et al.* (2010). "Characterization of a fibronectin-binding protein from *Lactobacillus casei* BL23." J Appl Microbiol **108**(3): 1050-1059.

Murga, M. a. L. F., G. Font de Valdez*, et al.* (2000). "Changes in the Surface Potential of *Lactobacillus acidophilus* under Freeze–Thawing Stress." Cryobiology **41**(1): 10-16.

Nagpal, R., A. Kumar*, et al.* (2012). "Probiotics, their health benefits and applications for developing healthier foods: a review." FEMS Microbiology Letters **334**(1): 1-15.

Nakayama, J., A. D. Akkermans*, et al.* (2003). "High-throughput PCR screening of genes for three-component regulatory system putatively involved in quorum sensing from low-G + C gram-positive bacteria." Biosci Biotechnol Biochem **67**(3): 480-489.

Nes, I. F., D. B. Diep*, et al.* (1996). "Biosynthesis of bacteriocins in lactic acid bacteria." Antonie Van Leeuwenhoek **70**(2-4): 113-128.

Neuhaus, F. C. (1985). "Inter-chain transacylation of D-alanine ester residues of lipoteichoic acid: a unique mechanism of membrane communication." Biochem Soc Trans **13**(6): 987-990.

Neuhaus, F. C. and J. Baddiley (2003). "A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria." Microbiol. Mol. Biol. Rev. **67**(4): 686-723.

Neuhaus, F. C., M. P. Heaton*, et al.* (1996). "The *dlt* operon in the biosynthesis of D-alanyl-lipoteichoic acid in *Lactobacillus casei*." Microb. Drug Resist. **2**(1): 77-84.

Neumüller, A. M., D. Konz*, et al.* (2001). "The two-component regulatory system BacRS is associated with bacitracin 'self-resistance' of *Bacillus licheniformis* ATCC 10716." Eur J Biochem **268**(11): 3180-3189.

Neville, B. A., B. M. Forde*, et al.* (2012). "Characterization of pro-inflammatory flagellin proteins produced by *Lactobacillus ruminis* and related motile *Lactobacilli*." PLoS ONE **7**(7): e40592.

Nicholson, J. K., E. Holmes*, et al.* (2012). "Host-Gut Microbiota Metabolic Interactions." Science **336**(6086): 1262-1267.

Ntamere, A. S., D. J. Taron*, et al.* (1987). "Assembly of D-alanyl-lipoteichoic acid in *Lactobacillus casei*: mutants deficient in the D-alanyl ester content of this amphiphile." J Bacteriol **169**(4): 1702-1711.

Ohki, R., Giyanto*, et al.* (2003). "The BceRS two-component regulatory system induces expression of the bacitracin transporter, BceAB, in *Bacillus subtilis*." Mol. Microbiol. **49**(4): 1135-1144.

Okuda, K.-i., Y. Aso*, et al.* (2008). "Cooperative Transport between NukFEG and NukH in Immunity against the Lantibiotic Nukacin ISK-1 Produced by *Staphylococcus warneri* ISK-1." J Bacteriol **190**(1): 356-362.

Otto, M., A. Peschel*, et al.* (1998). "Producer self-protection against the lantibiotic epidermin by the ABC transporter EpiFEG of *Staphylococcus epidermidis* Tü3298." FEMS Microbiol Lett **166**(2): 203-211.

Ou, L. T. and R. E. Marquis (1970). "Electromechanical interactions in cell walls of gram-positive cocci." J. Bacteriol. **101**(1): 92-101.

Ouhara, K., H. Komatsuzawa*, et al.* (2008). "Increased resistance to cationic antimicrobial peptide LL-37 in methicillin-resistant strains of *Staphylococcus aureus*." Journal of Antimicrobial Chemotherapy **61**(6): 1266-1269.

Ouwehand, A. C., Vaughan, Elaine E. (2006). Gastrointestinal Microbiology, Taylor & Francis Group.

Pal, M. K., T. C. Ghosh*, et al.* (1990). "Studies on the conformation of and metal ion binding by teichoic acid of *Staphylococcus aureus*." Biopolymers **30**(3-4): 273-277.

Palomino, M. M., M. C. Allievi*, et al.* (2013). "Osmotic stress adaptation in *Lactobacillus casei BL23* leads to structural changes in the cell wall polymer lipoteichoic acid." Microbiology **159**(Pt 11): 2416-2426.

Palumbo, E., M. Deghorain*, et al.* (2006). "D-alanyl ester depletion of teichoic acids in *Lactobacillus plantarum* results in a major modification of lipoteichoic acid composition and cell wall perforations at the septum mediated by the Acm2 autolysin." J. Bacteriol. **188**(10): 3709-3715.

Perea Vélez, M., T. L. Verhoeven*, et al.* (2007). "Functional analysis of D-alanylation of lipoteichoic acid in the probiotic strain *Lactobacillus rhamnosus* GG." Appl Environ Microbiol **73**(11): 3595-3604.

Perego, M., P. Glaser*, et al.* (1995). "Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*: identification of genes and regulation." J. Biol. Chem. **270**(26): 15598-15606.

Peschel, A. (2002). "How do bacteria resist human antimicrobial peptides?" Trends Microbiol. **10**(4): 179-186.

Peschel, A., R. W. Jack*, et al.* (2001). "*Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine." J Exp Med **193**(9): 1067-1076.

Peschel, A., M. Otto*, et al.* (1999). "Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides." J. Biol. Chem. **274**(13): 8405-8410.

Peschel, A. and H. G. Sahl (2006). "The co-evolution of host cationic antimicrobial peptides and microbial resistance." Nat. Rev. Microbiol. **4**(7): 529-536.

Peschel, A., C. Vuong*, et al.* (2000). "The D-alanine residues of *Staphylococcus aureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes." Antimicrob Agents Chemother **44**(10): 2845-2847.

Pfeiler, E. A., M. A. Azcarate-Peril*, et al.* (2007). "Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*." J Bacteriol **189**(13): 4624-4634.

Pietiäinen, M., P. Francois*, et al.* (2009). "Transcriptome analysis of the responses of *Staphylococcus aureus* to antimicrobial peptides and characterization of the roles of vraDE and vraSR in antimicrobial resistance." BMC Genomics **10**: 429.

Piuri, M., C. Sanchez-Rivas*, et al.* (2005). "Cell wall modifications during osmotic stress in *Lactobacillus casei*." J Appl Microbiol **98**(1): 84-95.

Podlesek, Z., A. Comino*, et al.* (1995). "*Bacillus licheniformis* bacitracin-resistance ABC transporter: relationship to mammalian multidrug resistance." Mol Microbiol **16**(5): 969-976.

Poolman, B., D. Molenaar*, et al.* (1991). "Malolactic fermentation: electrogenic malate uptake and malate/lactate antiport generate metabolic energy." J Bacteriol **173**(19): 6030-6037.

Poupard, J. A., I. Husain*, et al.* (1973). "Biology of the bifidobacteria." Bacteriol Rev **37**(2): 136-165.

Poyart, C., E. Pellegrini*, et al.* (2003). "Attenuated virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells." Mol Microbiol **49**(6): 1615-1625.

Qin, J., R. Li*, et al.* (2010). "A human gut microbial gene catalogue established by metagenomic sequencing." Nature **464**(7285): 59-65.

Quevedo, B., E. Giertsen*, et al.* (2011). "Phylogenetic group- and species-specific oligonucleotide probes for single-cell detection of lactic acid bacteria in oral biofilms." BMC Microbiol **11**(1): 14.

Ra, R., M. M. Beerthuyzen*, et al.* (1999). "Effects of gene disruptions in the nisin gene cluster of *Lactococcus lactis* on nisin production and producer immunity." Microbiology **145**: 1227-1233.

Raviv, Y., H. B. Pollard*, et al.* (1990). "Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells." Journal of Biological Chemistry **265**(7): 3975-3980.

Renault, P., C. Gaillardin*, et al.* (1988). "Role of malolactic fermentation in lactic acid bacteria." Biochimie **70**(3): 375-379.

Revilla-Guarinos, A., C. Alcantara*, et al.* (2014). "Characterization of the response to low pH of Lactobacillus casei DeltaRR12, a mutant strain with low D-alanylation activity and sensitivity to low pH." J Appl Microbiol **116**(5): 1250-1261.

Revilla-Guarinos, A., S. Gebhard*, et al.* (2013). Characterization of a Regulatory Network of Peptide Antibiotic Detoxification Modules in *Lactobacillus casei* BL23. Appl. Environ. Microbiol.

Revilla-Guarinos, A., S. Gebhard*, et al.* (2014). "Defence against antimicrobial peptides: different strategies in Firmicutes." Environ Microbiol **16**(5): 1225-1237.

Richet, E., A. L. Davidson*, et al.* (2012). "The ABC transporter $MalFGK_2$ sequesters the MalT transcription factor at the membrane in the absence of cognate substrate." Mol. Microbiol. **85**(4): 632-647.

Rietkötter, E., D. Hoyer*, et al.* (2008). "Bacitracin sensing in *Bacillus subtilis*." Mol. Microbiol. **68**(3): 768-785.

Risoen, P. A., L. S. Havarstein*, et al.* (1998). "Identification of the DNA-binding sites for two response regulators involved in control of bacteriocin synthesis in *Lactobacillus plantarum* C11." Mol Gen Genet **259**(2): 224-232.

Rivas-Sendra, A., J. M. Landete*, et al.* (2011). "Response of *Lactobacillus casei* BL23 to phenolic compounds." J. Appl. Microbiol. **111**(6): 1473-1481.

Rizzo, A. F., H. Korkeala*, et al.* (1987). "Gas chromatography analysis of cellular fatty acids and neutral monosaccharides in the identification of lactobacilli." Appl Environ Microbiol **53**(12): 2883-2888.

Robles Alonso, V. and F. Guarner (2013). "Linking the gut microbiota to human health." British Journal of Nutrition **109**(SupplementS2): S21-S26.

Rochat, T., L. Bermudez-Humaran*, et al.* (2007). "Anti-inflammatory effects of *Lactobacillus casei* BL23 producing or not a manganese-dependant catalase on DSS-induced colitis in mice." Microb Cell Fact **6**: 22.

Rode, T. M., T. Møretrø*, et al.* (2010). "Responses of *Staphylococcus aureus* exposed to HCl and organic acid stress." Can J Microbiol **56**(9): 777-792.

Rowley, G., M. Spector*, et al.* (2006). "Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens." Nat Rev Microbiol **4**(5): 383-394.

Ruiz, N. and T. J. Silhavy (2005). "Sensing external stress: watchdogs of the *Escherichia coli* cell envelope." Curr Opin Microbiol **8**(2): 122-126.

Rytömaa, M., P. Mustonen*, et al.* (1992). "Reversible, nonionic, and pH-dependent association of cytochrome *c* with cardiolipin-phosphatidylcholine liposomes." J Biol Chem **267**(31): 22243-22248.

Saar-Dover, R., A. Bitler*, et al.* (2012). "D-alanylation of lipoteichoic acids confers resistance to cationic peptides in group B *streptococcus* by increasing the cell wall density." PLoS Pathog **8**(9): e1002891.

Saavedra, J. M. (2001). "Clinical applications of probiotic agents." The American Journal of Clinical Nutrition **73**(6): 1147S-1151S.

Saïd-Salim, B., P. M. Dunman*, et al.* (2003). "Global regulation of *Staphylococcus aureus* genes by Rot." J Bacteriol **185**(2): 610-619.

Sanders, M. E. (2003). "Probiotics: Considerations for Human Health." Nutrition Reviews **61**(3): 91-99.

Sanders, M. E., F. Guarner*, et al.* (2013). "An update on the use and investigation of probiotics in health and disease." Gut **62**(5): 787-796.

Savage, D. C. (1977). "Microbial ecology of the gastrointestinal tract." Annu Rev Microbiol **31**: 107-133.

Scharf, B. E. (2010). "Summary of useful methods for two-component system research." Curr Opin Microbiol **13**(2): 246-252.

Scherer, K., I. Wiedemann*, et al.* (2013). "Aggregates of nisin with various bactoprenol-containing cell wall precursors differ in size and membrane permeation capacity." Biochim. Biophys. Acta (BBA) - Biomembranes **1828**(11): 2628-2636.

Schiffrin, E. J., F. Rochat*, et al.* (1995). "Immunomodulation of human blood cells following the ingestion of lactic acid bacteria." J Dairy Sci **78**(3): 491-497.

Schneider, T., T. Kruse*, et al.* (2010). "Plectasin, a fungal defensin, targets the bacterial cell wall precursor Lipid II." Science **328**(5982): 1168-1172.

Schneider, T. and H.-G. Sahl (2010). "An oldie but a goodie: cell wall biosynthesis as antibiotic target pathway." Int. J. Med. Microbiol. **300**(2–3): 161-169.

Schramm, M., V. Klybas*, et al.* (1958). "Phosphorolytic cleavage of fructose-6-phosphate by fructose-6-phosphate phosphoketolase from Acetobacter xylinum." J Biol Chem **233**(6): 1283-1288.

Schrecke K., Staroń A.*, et al.* (2012). Two-component Signaling in the Gram-positive Envelope Stress Response: Intramembrane-sensing Histidine Kinases and Accessory Membrane Proteins. Two-component Systemes in Bacteria. Book. G. Roy Gross and Dagmar Beier University of Würzburg, Caister Academic Press.

Schultz, M., C. Veltkamp*, et al.* (2002). "Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice." Inflamm Bowel Dis **8**(2): 71-80.

Sengupta, R., E. Altermann*, et al.* (2013). "The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract." Mediators Inflamm **2013**: 237921.

Shaaly, A., F. Kalamorz*, et al.* (2013). "Undecaprenyl pyrophosphate phosphatase confers low-level resistance to bacitracin in *Enterococcus faecalis*." J. Antimicrob. Chemother. **68**(7): 1583-1593.

Sheng, J. and R. E. Marquis (2007). "Malolactic fermentation by *Streptococcus mutans*." FEMS Microbiology Letters **272**(2): 196-201.

Siegumfeldt, H., K. Björn Rechinger*, et al.* (2000). "Dynamic Changes of Intracellular pH in Individual Lactic Acid Bacterium Cells in Response to a Rapid Drop in Extracellular pH." Appl Environ Microbiol **66**(6): 2330-2335.

Siewert, G. and J. L. Strominger (1967). "Bacitracin: an inhibitor of the dephosphorylation of lipid pyrophosphate, an intermediate in the biosynthesis of the peptidoglycan of bacterial cell walls." Proc Natl Acad Sci U S A **57**(3): 767-773.

Spano, G. and S. Massa (2006). "Environmental Stress Response in Wine Lactic Acid Bacteria: Beyond *Bacillus subtilis*." Critical Reviews in Microbiology **32**(2): 77-86.

Staroń, A., D. E. Finkeisen*, et al.* (2011). "Peptide antibiotic sensing and detoxification modules of *Bacillus subtilis*." Antimicrob. Agents Chemother. **55**(2): 515-525.

Stein, T., S. Heinzmann*, et al.* (2005). "Expression and functional analysis of the subtilin immunity genes *spaIFEG* in the subtilin-sensitive host *Bacillus subtilis* MO1099." J Bacteriol **187**(3): 822-828.

Stein, T., S. Heinzmann*, et al.* (2003). "Function of Lactococcus lactis Nisin Immunity Genes nisI and nisFEG after Coordinated Expression in the Surrogate Host Bacillus subtilis." Journal of Biological Chemistry **278**(1): 89-94.

Stein, T., S. Heinzmann*, et al.* (2003). "Function of *Lactococcus lactis* nisin immunity genes *nisI* and *nisFEG* after coordinated expression in the surrogate host *Bacillus subtilis*." J Biol Chem **278**(1): 89-94.

Stock, A. M., V. L. Robinson*, et al.* (2000). "Two-component signal transduction." Annu Rev Biochem **69**: 183-215.

Storm, D. R. and J. L. Strominger (1973). "Complex formation between bacitracin peptides and isoprenyl pyrophosphates. The specificity of lipid-peptide interactions." J Biol Chem **248**(11): 3940-3945.

Sturme, M. H. J., J. Nakayama*, et al.* (2005). "An agr-Like Two-Component Regulatory System in *Lactobacillus plantarum* Is Involved in Production of a Novel Cyclic Peptide and Regulation of Adherence." J Bacteriol **187**(15): 5224-5235.

Swoboda, J. G., J. Campbell*, et al.* (2010). "Wall teichoic acid function, biosynthesis, and inhibition." ChemBioChem **11**(1): 35-45.

Takala, T. M., O. Koponen*, et al.* (2004). "Lipid-free NisI: interaction with nisin and contribution to nisin immunity via secretion." FEMS Microbiology Letters **237**(1): 171-177.

Takala, T. M. and P. E. Saris (2006). "C terminus of NisI provides specificity to nisin." Microbiology **152**(Pt 12): 3543-3549.

Thedieck, K., T. Hain*, et al.* (2006). "The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*." Mol. Microbiol. **62**(5): 1325-1339.

Tissier, H. (1907). Traitement des infections intestinales par la méthode de transformation de la flore bactérienne de l'intestin. S.l., s.n.].

Toh, H., K. Oshima*, et al.* (2013). "Genomic Adaptation of the *Lactobacillus casei* Group." PLoS ONE **8**(10): e75073.

Tursi, A., G. Brandimarte*, et al.* (2010). "Treatment of relapsing mild-to-moderate ulcerative colitis with the probiotic VSL#3 as adjunctive to a standard pharmaceutical treatment: a double-blind, randomized, placebo-controlled study." Am J Gastroenterol **105**(10): 2218-2227.

Tymczyszyn, E. E., M. Del Rosario Díaz*, et al.* (2007). "Volume recovery, surface properties and membrane integrity of *Lactobacillus delbrueckii* subsp. *bulgaricus* dehydrated in the presence of trehalose or sucrose." J Appl Microbiol **103**(6): 2410-2419.

Valyasevi, R., W. E. Sandine*, et al.* (1990). "The bacteriophage kh receptor of *Lactococcus lactis* subsp. *cremoris* KH is the rhamnose of the extracellular wall polysaccharide." Appl Environ Microbiol **56**(6): 1882-1889.

van de Guchte, M., P. Serror*, et al.* (2002). "Stress responses in lactic acid bacteria." Antonie Van Leeuwenhoek **82**(1-4): 187-216.

van Heijenoort, J. (2007). "Lipid intermediates in the biosynthesis of bacterial peptidoglycan." Microbiol. Mol. Biol. Rev. **71**(4): 620-635.

Vélez, M. P., S. C. J. De Keersmaecker*, et al.* (2007). "Adherence factors of *Lactobacillus* in the human gastrointestinal tract." FEMS Microbiology Letters **276**(2): 140-148.

von Schillde, M.-A., G. Hörmannsperger*, et al.* (2012). "Lactocepin Secreted By *Lactobacillus* Exerts Anti-Inflammatory Effects By Selectively Degrading Proinflammatory Chemokines." Cell Host & Microbe **11**(4): 387-396.

Waddington, L., T. Cyr*, et al.* (2010). "Understanding the acid tolerance response of bifidobacteria." J Appl Microbiol **108**(4): 1408-1420.

Walter, J. and R. Ley (2011). "The human gut microbiome: ecology and recent evolutionary changes." Annu. Rev. Microbiol. **65**: 411-429.

Walter, J., D. M. Loach*, et al.* (2007). "D-alanyl ester depletion of teichoic acids in *Lactobacillus reuteri* 100-23 results in impaired colonization of the mouse gastrointestinal tract." Environ Microbiol **9**(7): 1750-1760.

Watterlot, L., T. Rochat*, et al.* (2010). "Intragastric administration of a superoxide dismutase-producing recombinant *Lactobacillus casei* BL23 strain attenuates DSS colitis in mice." Int J Food Microbiol **144**(1): 35-41.

Wecke, J., K. Madela*, et al.* (1997). "The absence of D-alanine from lipoteichoic acid and wall teichoic acid alters surface charge, enhances autolysis and increases susceptibility to methicillin in *Bacillus subtilis*." Microbiology **143**: 2953-2960.

Weidenmaier, C. and A. Peschel (2008). "Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions." Nat. Rev. Micro. **6**(4): 276-287.

Wiedemann, I., E. Breukink*, et al.* (2001). "Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity." J. Biol. Chem. **276**(3): 1772-1779.

Wilson, W. W., M. M. Wade*, et al.* (2001). "Status of methods for assessing bacterial cell surface charge properties based on zeta potential measurements." J Microbiol Methods **43**(3): 153-164.

Winkler, M. E. and J. A. Hoch (2008). "Essentiality, bypass, and targeting of the YycFG (VicRK) two-component regulatory system in gram-positive bacteria." J Bacteriol **190**(8): 2645-2648.

Wu, C., J. Huang*, et al.* (2013). "Progress in engineering acid stress resistance of lactic acid bacteria." Appl Microbiol Biotechnol: 1-9.

Wu, C., J. Zhang*, et al.* (2012). "*Lactobacillus casei* combats acid stress by maintaining cell membrane functionality." J Ind Microbiol Biotechnol **39**(7): 1031-1039.

Zhang, Y. M. and C. O. Rock (2008). "Membrane lipid homeostasis in bacteria." Nat Rev Microbiol **6**(3): 222-233.

Zoetendal, E. G., A. von Wright*, et al.* (2002). "Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces." Appl Environ Microbiol **68**(7): 3401-3407.

OBJECTIVES

No es porque las cosas son difíciles que no osamos hacerlas,
es porque no osamos hacerlas que son difíciles.

Séneca

OBJECTIVES

Lactobacillus casei is a probiotic strain that must survive to a variety of stress conditions in order to arrive to the intestine in a viable and active state. Two Component Systems (TCS) are signal transduction mechanisms broadly characterized in the stress response in model and pathogenic microorganisms. However, the information about the possible role of TCS in the physiology of *L. casei* strains is scarce. This study is part of a research line focused on the study of the role of TCS in the general physiology and stress response of *L. casei* BL23, as well as the determination of the mechanisms activated to respond to specific environmental stresses.

For this study the following objectives were proposed:

1. Identification and characterization of TCS of *L. casei* and their involvement in its general physiology and stress response.
2. Determination of the role of *L. casei* BL23 TCS09 and TCS12 in the antimicrobial peptide (AMPs) stress response.
3. Study of the involvement of *L. casei* BL23 TCS12 in the acid stress response of this organism.

CHAPTER 1

Nothing is ever as simple as it first seems.

C´est le temps que tu as perdu pour ta rose
qui fait ta rose si importante.

Le Petit Prince

CHAPTER 1

PART I

Influence of Two-Component Signal Transduction Systems of *Lactobacillus casei* BL23 on Tolerance to Stress Conditions

Cristina Alcántara, **Ainhoa Revilla-Guarinos** and Manuel Zúñiga

Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC) PO Box 73, 46100 Burjassot, Valencia, Spain

RUNNING TITLE: Two component systems in stress tolerance of *L. casei*

Published in **Applied and Environmental Microbiology** (2011) **77**: 1516-1519.

ABSTRACT

L. casei BL23 encodes 17 two-component signal transduction systems. Insertional mutations were introduced into each gene encoding the cognate response regulators and their effect on growth under different conditions assayed. Inactivation of systems TC01, TC06 and TC12; (LCABL_02080-LCABL_02090, LCABL_12050-LCABL_12060 and LCABL_19600-LCABL_19610) led to major growth defects under the conditions assayed.

INTRODUCTION

Lactobacillus casei is a facultative heterofermentative lactic acid bacterium used in the food industry as a starter culture for milk fermentation, for maturation of cheeses and as probiotic (de Vrese and Schrezenmeir, 2008). Probiotic microorganisms must survive the industrial production processes (Corcoran *et al.*, 2008; Santivarangkna *et al.*, 2008) and transit through the gastrointestinal tract (Corcoran *et al.*, 2008). Bacteria have evolved sophisticated mechanisms to detect and adapt to environmental changes, and among them, two-component systems (TCS) play a central role (Stock *et al.*, 2000). TCS typically consist of a sensor kinase (HK) and a response regulator (RR) (Stock *et al.*, 2000). HKs monitor environmental signals and, in response to a stimulus, autophosphorylate and subsequently transfer the phosphoryl group to the RR, thus modulating its activity.

The role of TCS in *Lactobacillus* is not well understood, although they are likely involved in quorum sensing, production of bacteriocins (Risoen *et al.*, 1998; Sturme *et al.*, 2007; Fujii *et al.*, 2008; Maldonado-Barragan *et al.*, 2009), and possibly in the stress response (Morel-Deville *et al.*, 1998; Azcarate-Peril *et al.*, 2005; Pfeiler *et al.*, 2007). The availability of complete genome sequences of two *L. casei* strains (Makarova *et al.*, 2006; Mazé *et al.*, 2010) enables a more comprehensive study of the role of TCS in the stress response of this organism. In this study, we used a broad and operational definition of stress, as follows: any deviation from optimal growth conditions that results in a reduced growth rate or lower level of biomass (Hazen and Stahl, 2006).

MATERIALS AND METHODS

The genome sequences of ***L. casei*** strains BL23 and ATCC 334 harbor 17 putative TCS. For simplicity, we have numerically renamed them TC01 to TC17 (see Table S1 in the supplemental material). TC17 corresponds to the previously characterized system MaeKR (Landete *et al.*, 2010). The strains and plasmids used in this study are listed in Table S2 in the supplemental material. The primers used are listed in Table S3 in the supplemental material. Insertion mutants were obtained by cloning internal DNA fragments of each RR-encoding gene in plasmid pRV300 (Leloup *et al.*, 1997) and introduced in ***L. casei*** BL23 by electroporation (Posno *et al.*, 1991). BL23-derivative strains harboring complete deletions of the LCABL_02080 (RR01), LCABL_12050 (RR06), and LCABL_19600 (RR12) genes were also obtained by insertion of plasmid pRV300 harboring the regions immediately upstream and downstream of each target gene and subsequent internal recombination. Complementation of the RR01 and RR06 deletions was achieved by cloning of the corresponding genes into the expression vector pT1NX (Schotte *et al.*, 2000).

The growth of ***L. casei*** BL23 and that of its derivative RR defective mutants under reference conditions (MRS at 37°C without shaking) and using MRS supplemented with 0.5% bile, MRS supplemented with 0.6 M NaCl, MRS adjusted to pH 3.75, and MRS at 42°C were compared. Growth was monitored by changes in the optical density at 595 nm (OD_{595}) in a microtiter plate reader. At least three independent replicates of each growth curve were obtained. Maximal growth rates (μ_{max}) and the increment in OD values were considered to compare the performance of ***L. casei*** BL23 and its derivative mutants. Significant differences in growth parameters under the reference condition between the wild-type strain and each of the mutants were determined by one-way analysis of variance (ANOVA). Levene's test was used to assess the equality of error variances. To determine whether the responses of the mutant strains to each stress condition assayed were significantly different from that of the wild type, pairwise two-way ANOVA analyses were performed, testing the growth of ***L. casei*** BL23 and that of each mutant strain under the reference condition and each of the other stress conditions. We considered a significant

difference to be detected if the analysis estimated that both the strain variable and interaction were below P values of 0.01.

Resistance to vancomycin, bacitracin, gramicidin, or nisin was determined in MRS using serial dilutions of the antimicrobial agents. The assays were performed in 96-well microtiter plates incubated for 24 h. The MIC (expressed as the number of μg ml^{-1}) was defined as the lowest concentration of the antimicrobial agent needed to totally inhibit the growth of the bacterial strain. The 50% inhibitory concentration (IC_{50}) was considered the concentration of the antimicrobial agent that diminished the maximal growth rate (μ_{max}) to 50% of its value under reference growth conditions.

RESULTS & DISCUSSION

Insertion mutants were obtained for each RR, thus indicating that none of the TCS are essential for growth. RR16 is homologous to RR YycF/VicR, which is essential for growth in other low-G+C-content Gram-positive bacteria (Winkler and Hoch, 2008). Inactivation of the YycF homolog-encoding gene (rrp-3) in *Lactobacillus sakei* did not result in any significant differences with the parental strain under a number of stress conditions (Morel-Deville *et al.*, 1998). This suggests that the YycFG TCS is not essential in lactobacilli, although it is in the closely related enterococci and streptococci.

The growth rates of the different mutants were similar to that of the wild-type strain under reference conditions, except those of the TC04 and TC11 mutants, which were significantly reduced, and TC12, in which the maximum cell density was significantly lower than that of the wild-type strain (Fig. 1A; see also Table S4 in the supplemental material). The inactivation of systems TC01, TC06, and TC12 led to major growth defects under stress conditions (Fig. 1 and Table 1; see also Tables S5A and B in the supplemental material).

Table 1. Results of the growth assays carried out with *L. casei* BL23 and selected TCS-defective mutants under different growth conditions[a].

Condition	Growth parameter	Result[b]			
		BL23	TC01	TC06	TC12
Bile, 0.5%	μ_{max}	0.17±<10^{-2}	NG[b]	NG	0.08±<10^{-2}
	Change in OD	1.15±0.14	NG	NG	0.52±0.03
0.6 M NaCl	μ_{max}	0.23±0.01	NG	NG	0.21±0.01
	Change in OD	1.83±0.04	NG	NG	1.81±0.03
pH 3.75	μ_{max}	0.07±<10^{-2}	0.03±<10^{-2}	0.03±0.01	NG
	Change in OD	0.56±0.07	0.19±0.06	0.14±0.01	NG
42°C	μ_{max}	0.26±0.01	0.24±<10^{-2}	0.17±0.01	0.22±0.02
	Change in OD	2.19±0.10	2.07±0.15	0.46±0.03	1.02±0.05

[a] See Tables S4A and B in the supplemental material for data of all mutants and the results of the ANOVA.
[b] The data shown are means and standard deviations. NG, no growth.

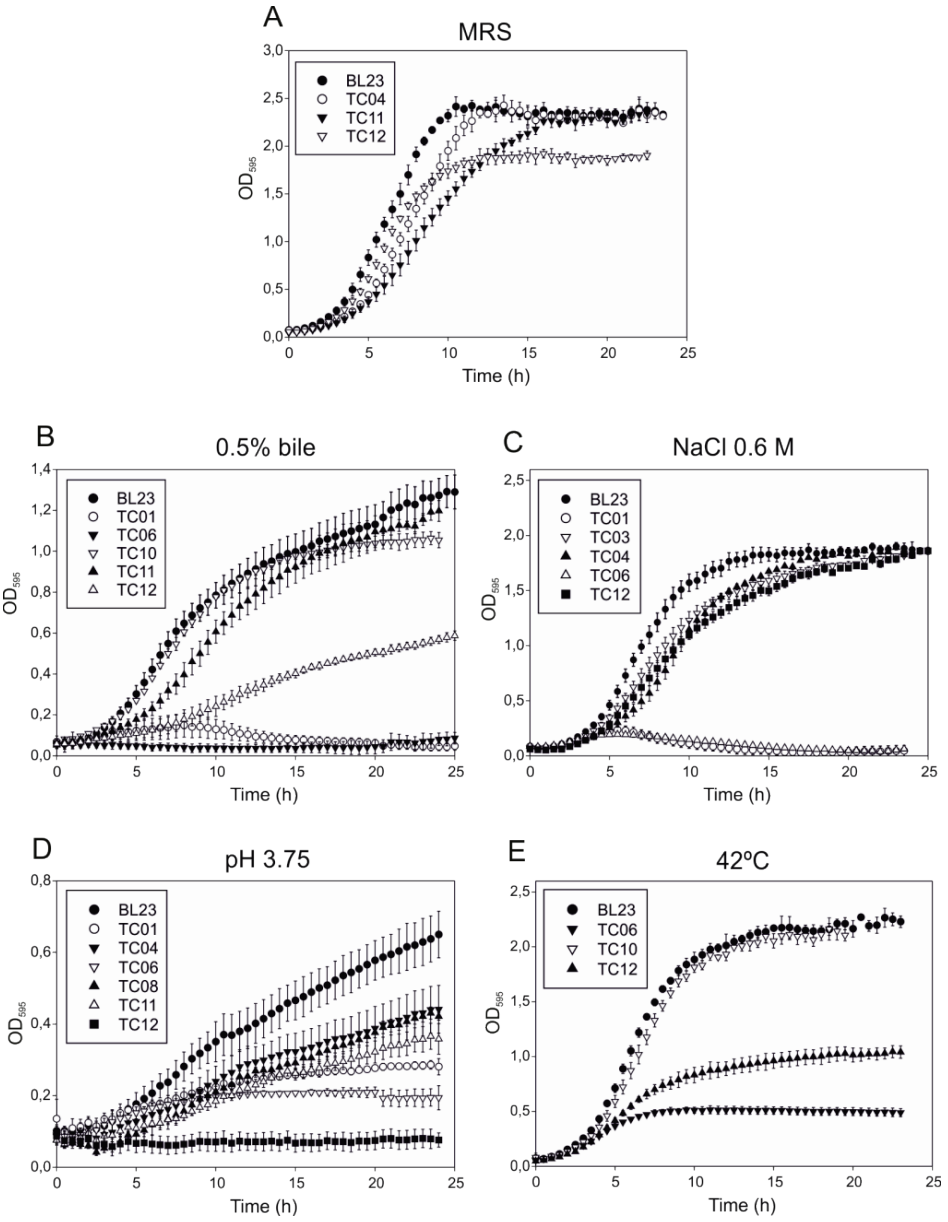


Figure 1.Growth of *L. casei* BL23 and TCS-defective mutants under different conditions (only strains that displayed significant differences with strain BL23 are shown). Error bars indicate standard deviations (at least three replicates).

The effect of the inactivation of *L. casei* TCS on tolerance against antibiotics targeted to the cell envelope was also investigated. BL23 and its derivative mutants were resistant to gramicidin and vancomycin, but IC_{50}s for vancomycin in the TC01, TC06, and TC12 mutants were lower than that in the parental strain (see Table S6 in the supplemental material). In contrast, *L. casei* BL23 was sensitive to bacitracin and nisin (see Table S6), and the responses of the TCS mutants varied. The TC01, TC09, and TC10 mutants were more sensitive to both of the antimicrobials than BL23, whereas three other mutants (TC06, TC11, and TC12) were more sensitive to nisin only. Three mutants were more resistant to bacitracin (TC15, TC16, and TC17), and one mutant was more resistant to both of the antimicrobials (TC04).

To determine the possible polar effects of the insertional inactivation of systems TC01, TC06, and TC12, strains carrying deletions of RR01, RR06, and RR12 (ΔRR01, ΔRR06, and ΔRR12, respectively) and the corresponding complemented strains (except for the ΔRR12 mutant, which was impervious to transformation) were obtained. BL23 and ΔRR01 strains grew similarly under reference conditions. The growth of the ΔRR01 mutant under different stress conditions was similar to that observed for the insertional mutant (see Fig. S1A to D and Table S6 in the supplemental material), and the effects of the mutation were relieved in the complemented strain ΔRR01-c, except in the presence of 0.6 M NaCl, where the ΔRR01 mutant was able to grow (see Fig. S1B in the supplemental material). Therefore, the growth defect observed with salt was possibly due to a polar effect on the expression of some of the genes located downstream. TCS01 is homologous to the rrp-31 hpk-31 (LSA0277-78) system of *L. sakei* and the CroRS system of *Enterococcus faecalis* (Comenge *et al.*, 2003; Le Breton *et al.*, 2003; Le Breton *et al.*, 2007). Inactivation of the cognate RR in *L. sakei* led to premature arrest of growth under reference conditions (MRS at 30°C), poor growth at a high temperature (39°C), sensitivity to heat shock, aeration, H_2O_2, and higher resistance to vancomycin (Morel-Deville *et al.*, 1998). These results contrast with our observations of the ΔRR01 mutant, thus suggesting that these homologous TCS have different physiological roles. The sensitivity of the ΔRR01 mutant to bile and the cell envelope-targeted antimicrobials bacitracin, nisin and vancomycin suggests that TCS01 is involved in cell envelope stress tolerance.

The ΔRR06 and ΔRR12 mutants showed phenotypes very similar to those of their corresponding insertional mutant strains (see Fig. S2 and Table S6 in the supplemental material). Complementation of the ΔRR06 mutant with pT1-RR06 relieved the effects of the mutation, and we conclude that the effects observed were due to the inactivation of RR06 and not to polar effects on downstream genes. Though complementation of the ΔRR12 mutant was not achieved, the similarity of the phenotypes of TC12 and ΔRR12 strains suggests that the observed effects are due mainly to the inactivation of RR12.

TCS06 is homologous to the *Bacillus subtilis* YclJ-YclK TCS, which is activated under oxygen limitation conditions (Kobayashi *et al.*, 2001), and *E. faecalis* system Err06-Ehk06. Inactivation of this system in *E. faecalis* V583 resulted in a heat- and sodium dodecyl sulfate (SDS)-sensitive phenotype (Hancock and Perego, 2004). Furthermore, it has been shown that Err06 is involved in resistance to H_2O_2 in *E. faecalis* JH2-2 (Muller *et al.*, 2008). *L. casei* TC06 was very sensitive under most stress conditions assayed. In this sense, it is worth noting the presence of a putative transfer-messenger RNA (tmRNA)-carrying gene located upstream of the TCS06-encoding genes. Interestingly, the involvement of a homologous tmRNA of *Escherichia coli* in the cell envelope stress response has been recently demonstrated (Hobbs *et al.*, 2010), although both the involvement of TCS06 in the regulation of tmRNA expression and the actual role of this tmRNA remain to be established.

TCS12 is paralogous to TCS09, but in contrast to TC12, inactivation of TC09 resulted only in higher sensitivity to bacitracin and nisin than that of BL23 (see Table S6 in the supplemental material). TCS09 and TCS12 are homologous to the three paralogous TCS of *B. subtilis*, BceRS, YvcPQ, and YxdJK, involved in the cell envelope stress response (Jordan *et al.*, 2008). These three TCS are located next to genes encoding ABC transporters. Similarly, both TCS09 and TCS12 are located next to genes encoding putative ABC transporters. The lower resistances of TC09 against bacitracin and nisin and of TC12 against nisin suggest that these systems may also be involved in the cell envelope stress response. However, the growth defects of the TC12 mutant, particularly at a low pH, suggest that the functional role of this system in *L. casei* is quite different than that of its homolog, BceRS in *B. subtilis*.

In summary, this study shows that some TCS play a major role in the physiology of *L. casei* and its adaptation under changing environmental conditions. The detailed study of these systems should provide valuable insight into understanding the performance of this organism under conditions of industrial production and in the gastrointestinal habitat.

AKNOWLEDGMENTS

This work was financed by grants AGL2007-60975/ALI and Consolider Fun-C-Food CSD2007-00063 from the Spanish Ministerio de Ciencia e Innovación. A. Revilla-Guarinos is the recipient of an FPI grant (BES-2008-004527) from the Spanish Ministerio de Ciencia e Innovación.

We thank Laura Barrios and Fernando López for their help with statistical analyses, Amalia Blasco for technical assistance, and Vicente Monedero for critical reading of the manuscript.

SUPPLEMENTAL MATERIAL

Table S1.Histidine kinase and response regulators identified in *L. casei* genomes

System	Gene tag		Family	Most similar sequence[1]
	BL23	**ATCC334**		
TC01	LCABL_02090	LSEI_0220	IIIA	LSA0278 (*L. sakei*)
	LCABL_02080	LSEI_0219	OmpR	LSA0277 (*L. sakei*)
TC02	LCABL_05270	LSEI_0461	IIIA	RBAM_005810 (*B. amyloliquefaciens*)
	LCABL_05260	LSEI_0460	OmpR	CD0486 (*C. difficile*)
TC03	LCABL_07770	LSEI_0712	IIIA	LVIS_0355 (*L. brevis*)
	LCABL_07760	LSEI_0711	OmpR	llrF (*Lc. lactis*)
TC04	LCABL_10490	LSEI_0935	IIIA	LSA0501 (*L. sakei*)
	LCABL_10480	LSEI_0934	OmpR	LSA0500 (*L. sakei*)
TC05	LCABL_10650	LSEI_0951	IIIA	CLI_0968 (*C. botulinum*)
	LCABL_10640	LSEI_0950	OmpR	CLI_0967 (*C. botulinum*)
TC06	LCABL_12060	LSEI_1042	IIIA	LSA1214 (*L. sakei*)
	LCABL_12050	LSEI_1041	ompR	LVIS_1316 (*L. brevis*)
TC07	LCABL_14270	LSEI_1208	IIIA	CTC00159 (*C. tetani*)
	LCABL_14280	LSEI_1209	OmpR	SMU.1038c (*S. mutans*)
TC08	LCABL_14440	LSEI_1223	II	Lp_1943 (*L. plantarum*)
	LCABL_14430	LSEI_1222	NarL	Lp_1942 (*L. plantarum*)
TC09	LCABL_16420	LSEI_1419	IIIA	STER_1309 (*S. thermophilus*)
	LCABL_16430	LSEI_1420	OmpR	SAG0976 (*S. agalactiae*)
TC10	LCABL_18840	LSEI_1666	II	LSA1370 (*L. sakei*)
	LCABL_18830	LSEI_1665	NarL	EF2911 (*E. faecalis*)
TC11	LCABL_18970	LSEI_1678	IIIA	Hpk5 (*L. plantarum*)
	LCABL_18980	LSEI_1679	OmpR	LSA1384 (*L. sakei*)
TC12	LCABL_19610	LSEI_1741	IIIA	LSA1455 (*L. sakei*)
	LCABL_19600	LSEI_1740	OmpR	LSA1454 (*L. sakei*)
TC13	Pseudogene	Pseudo-gene		
	LCABL_25620	LSEI_2389	lytT	*pltR* (*L. plantarum*)
TC14	LCABL_27660	LSEI_2600		TDE0032 (*T. denticola*)
	LCABL_27650	LSEI _2599	lytT	CAC1581 (*C. acetobutylicum*)
TC15	LCABL_28710	LSEI _2680	IIIA	OEOE_0489 (*O. oeni*)
	LCABL_28720	LSEI_2681	OmpR	LP_0283 (*L. plantarum*)
TC16	LCABL_30120	LSEI_2807	IIIA	LSA0078 (*L. sakei*)
	LCABL_30130	LSEI_2808	OmpR	LSA0077 (*L. sakei*)
TC17	LCABL_30710	LSEI_2868	CitA	EF1209 (*E. faecalis*)
	LCABL_30720	LSEI_2869	CitB (IV)	*dpiA* (*S. pyogenes*)

[1] Sequences of *L. casei* or *Lactobacillus rhamnosus* are not considered.

Table S2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Ref. or source
E. coli DH5α	F⁻ *endA1 hsdR17 gyrA96 thi-1 recA1 relA1 supE44* Δ*lacU169* (Φ 80 *lacZ*ΔM15)	Stratagene
L. casei BL23	Wild type	B. Chassy, U. Illinois
L. casei TC01	LCABL_02080 mutant; pRV02080 Ery^r	This study
L. casei TC02	LCABL_05260 mutant; pRV05260 Ery^r	This study
L. casei TC03	LCABL_07760 mutant; pRV07760 Ery^r	This study
L. casei TC04	LCABL_10480 mutant; pRV10480 Ery^r	This study
L. casei TC05	LCABL_10640 mutant; pRV10640 Ery^r	This study
L. casei TC06	LCABL_12050 mutant; pRV12050 Ery^r	This study
L. casei TC07	LCABL_14280 mutant; pRV14280 Ery^r	This study
L. casei TC08	LCABL_14430 mutant; pRV14430Ery^r	This study
L. casei TC09	LCABL_16430 mutant; pRV16430 Ery^r	This study
L. casei TC10	LCABL_18830 mutant; pRV18830 Ery^r	This study
L. casei TC11	LCABL_18980 mutant; pRV18980 Ery^r	This study
L. casei TC12	LCABL_19600 mutant; pRV19600 Ery^r	This study
L. casei TC13	LCABL_25620 mutant; pRV25620 Ery^r	This study
L. casei TC14	LCABL_27650 mutant; pRV27650 Ery^r	This study
L. casei TC15	LCABL_28720 mutant; pRV28720 Ery^r	This study
L. casei TC16	LCABL_30130 mutant; pRV30130 Ery^r	This study
L. casei TC17	BL23 Δ*maeR*	BL315(Landete *et al.*, 2010)
L. casei ΔRR01	BL23 Δ*rrp11* (LCABL_2080)	This study
L. casei ΔRR01-c	BL23 Δ*rrp11* harbouring plasmid pT1-RR01	This study
L. casei ΔRR06	BL23 Δ*rrp7* (LCABL_12050)	This study
L. casei ΔRR06-c	BL23 Δ*rrp7* harbouring plasmid pT1-RR06	This study
L. casei ΔRR12	BL23 Δ*rrp1* (LCABL_19600)	This study
pRV300	Insertional vector for *Lactobacillus*, Amp^r, Ery^r	(Leloup *et al.*, 1997)
pRV02080	pRV300 containing a 372-pb internal fragment of LCABL_02080	This study
pRV05260	pRV300 containing a 368-pb internal fragment of LCABL_05260	This study
pRV07760	pRV300 containing a 369-pb internal fragment of LCABL_07760	This study
pRV10480	pRV300 containing a 399-pb internal fragment of LCABL_10480	This study
pRV10640	pRV300 containing a 367-pb internal fragment of LCABL_10640	This study
pRV12050	pRV300 containing a 422-pb internal fragment of LCABL_12050	This study
pRV14280	pRV300 containing a 406-pb internal fragment of LCABL_14280	This study
pRV14430	pRV300 containing a 347-pb internal fragment of LCABL_14430	This study

Table S2. *Continuation*

Strain or plasmid	Relevant characteristics[a]	Ref. or source
pRV16430	pRV300 containing a 354-pb internal fragment of LCABL_16430	This study
pRV18830	pRV300 containing a 349-pb internal fragment of LCABL_18830	This study
pRV18980	pRV300 containing a 378-pb internal fragment of LCABL_18980	This study
pRV19600	pRV300 containing a 382-pb internal fragment of LCABL_19600	This study
pRV25620	pRV300 containing a 373-pb internal fragment of LCABL_25620	This study
pRV27650	pRV300 containing a 365-pb internal fragment of LCABL_27650	This study
pRV28720	pRV300 containing a 383-pb internal fragment of LCABL_28720	This study
pRV30130	pRV300 containing a 364-pb internal fragment of LCABL_30130	This study
pRVRR01	pRV300 containing fused flanking fragments upstream and downstream of LCABL_02080	This study
pRVRR06	pRV300 containing fused flanking fragments upstream and downstream of LCABL_12050	This study
pRVRR12	pRV300 containing fused flanking fragments upstream and downstream of LCABL_19600	This study
pT1NX	Expression vector for Gram-positive bacteria harboring the constitutive P1 promoter, Ery^r	(Schotte *et al.*, 2000)
pT1-RR01	pT1NX with cloned LCABL_02080 (RR01) expressed from the P1 promoter	This study
pT1-RR06	pT1NX with cloned LCABL_12050 (RR06) and its promoter region	This study
pT1-RR12	pT1NX with cloned LCABL_19600 (RR12) and its promoter region	This study

[a] Amp^r, ampicillin resistance; Ery^r, erythromycin resistance.

Table S3. Oligonucleotides used in this study

Name	Secuence (5´→ 3´)[a]	Application
Lsei219RF	GATGCCTAATATGTCTGGGATG	Amplification of *rrp11* internal fragment (RR01)
Lsei219RR	CTCATCAGCCGAGAAAACAC	
Lsei460RF	CAACTTACCTGATACTGACG	Amplification of *spaR* internal fragment (RR02)
Lsei460RR	GTTCACGACTAAAGACTTGCC	
Lsei711RF	GCCAGAATTAAATGGTTTTGACG	Amplification of *llrF* internal fragment (RR03)
Lsei711RR	CCCACAAACGATCAAAAATCTG	
Lsei934RF	GCTGCCGAGTCTGAGCGGC	Amplification of *rrp2* internal fragment (RR04)
Lsei934RR	CCCCGTTCAATAAAGCATCTCG	
Lsei950RF	CTGAGCATGCCCGATGGTG	Amplification of LCABL_10640 internal fragment (RR05)
Lsei950RR	GCGTGTCCCACAAGGCTGC	
Lsei1041RF	TTTTGTCGACCTCAATCTGCCAAAAATGGAC	Amplification of *rrp7* internal fragment (RR06)
Lsei1041RR	TTTTCTGCAGCCAATTGCAGTAATTGTTCACG	
Lsei1209RF	GCTGCCAAACGTCACCGGTG	Amplification of LCABL_14280 internal fragment (RR07)
Lsei1209RR	GCGTTCATGAACGGCTCGCC	
Lsei1222RF	GAGATGCCCAAGTTAACCGG	Amplification of *rrp6* internal fragment (RR08)
Lsei1222RR	GTTCCTTCCGAGAGAAACAGC	
Lsei1420RF	CAGTCTGCCATATTTTAATGG	Amplification of LCABL_16430 internal fragment (RR09)
Lsei1420RR	CAGCGCTGAACAAAAGACTC	
Lsei1665RF	TTTTGTCGACGGATCYCGTGATGCCGGTG	Amplification of LCABL_18830 internal fragment (RR10)
Lsei1665RR	TTTTCTGCAGCTGTCGCAATTTCTTGATTCG	
Lsei1679RF	GATGTTGCCAGAATTAAACGG	Amplification of *rrp5* internal fragment (RR11)
Lsei1679RR	CGTCACGAGCCAAAACAACG	
Lsei1740RF	CCAGTTTTTGACGGCTATTATTGG	Amplification of *rrp1* internal fragment (RR12)
Lsei1740RR	GCCATAAGTCCTTTAATAACC	
Lsei2389RF	CCCTGGATCTTCTGTAAGTGG	Amplification of *pltR* internal fragment (RR13)
Lsei2389RR	GCAAAATGCTTCGATGACAGC	
Lsei2599RF	CAAGTTAGGTTCAGGCATGG	Amplification of LCABL_27650 internal fragment (RR14)
Lsei2599RR	CGTCGAGTTGAGGAAGAAAC	
Lsei2681RF	GGATGCTGCCTAAACTAGACG	Amplification of *rrp2* internal fragment (RR15)
Lsei2681RR	CAAGTCATCCCTCGAACAAGC	
Lsei2808RF	GATGTTACCAAAAATTGATGGG	Amplification of *rrp3* internal fragment (RR16)
Lsei2808RR	CAAGGTGTCGAGCAAGGTAG	
DEL_219AF	TTTTGTCGACGCGCAGTTCATTCAAATTACC	Amplification of *rr01up* fragment
DEL_219AR	GATTTTTTCGCTCATGATCGTGTATTGGCTCCGTTTATG	
DEL_219BF	CATAAACGGAGCCAATACACGATCATGAGCGAAAAA GTC	Amplification of *rr01down* fragment
DEL_219BR	TTTTCTGCAGCCTCAACGCGATAAAAACG	
PT219FOR	TTTTAGATCTCATAAACGGAGCCAATACAATG	Amplification of *rr01* gene
PT219REV	TTTTACTAGTCGTTAGGCCTCAACCTTATAG	
DEL1041AF	TTTTGTCGACGCTTTTGTCTGCTAGGTGAC	Amplification of *rr06up* fragment
DEL1041AR	GCTGGTAGAGCATTTTCATCGATGTACCCCTCATTTCA CAG	
DEL1041BR	TTTTCTGCAGGATGTTATCAAGATCCGTGC	Amplification of *rr06down* fragment
DEL1041BF	CTGTGAAATGAGGGGTACATCGATGAAAATGCTCTAC CAGC	
PT1041FOR	TTTTAGATCTGAAATGAGGGGTACATCATG	Amplification of *rr06* gene
PT1041REV	TTTTACTAGTGCATTTTCATCGATCAAGCC	

Table S3. *Continuation*

Name	Secuence (5´→ 3´)[a]	Application
CP1041FOR	TTTTGAATTCGATTAAGGTGCAAACGTTATG	Amplification of promoter region and *rr06* gene
CP1041REV	TTTTACTAGTATCGATCAAGCCCCGAATCATC	
DEL1740AF	TTTTAAGCTTCTTCCATATCAATAATTCGGTC	Amplification of *rr12up* fragment
DEL1740AR	GAAACCGCATCTGCCAGCCTCGATTTTAAACACGTGAC CAAC	
DEL1740BR	TTTTACTAGTGTACCTTGATTGATTACCGAGG	Amplification of *rr12down* fragment
DEL1740BF	GTTGGTCACGTGTTTAAAATCGAGGCTGGCAGATGCG GTTTC	
PT1740FOR	TTTTAGATCTGAAATGGAGTTGGTCACGTG	Amplification of *rr12* gene
PT1740REV	TTTTACTAGTCCAGCCTCCTAAGGAACG	
CP1740FOR	TTTTGAATTCATGCATGTGCTGCCAACTCC	Amplification of promoter region and *rr12* gene
CP1740REV	TTTTTCTAGATGCCAGCCTCCTAAGGAACG	
External primers		
C94-68754	CTAGCCCTGTTTTCTTAGGC	Screening of *rrp11* mutants (RR01)
C87-24906	CGTGTTGCAAGCGATTCCG	Screening of SpaR mutants (RR02)
C87-10203	GGATCATTATGAGAGTTACAGC	Screening of *llrF* mutants (RR03)
C85-48527	CAACCTATTGCCGCTTGCGG	Screening of *rrp2* mutants (RR04)
C85-65285	GACTATCCGGACTTTTTCGG	Screening of LCABL_10640 mutants (RR05)
C93-82083	GCAAGCCCATGATGCACTGCC	Screening of *rrp7* mutants (RR06)
C73-25455	GTGGCCTTGGGTGTGATGG	Screening of LCABL_14280 mutants (RR07)
C73-15384	CAACGTATGAAAGAAGTTAATGG	Screening of *rrp6* mutants (RR08)
C83-40666	CAGTTAGAATAGAAACTGTCC	Screening of LCABL_16430 mutants (RR09)
C68-7694	CTTGCAAATATCAAGGAACGTGC	Screening of LACBL_18830 mutants (RR10)
C92-145449	GTCAAAATGAGAAAACTGTGC	Screening of *rrp5* mutants (RR11)
C92-59224	CCTAAAAGCCGGACAAACCC	Screening of *rrp1* mutants (RR12)
C89-64634	GCACGGCAATGGCTTGACC	Screening of *pltR* mutants (RR13)
C96-12266	GCTGACTCACTCTAGTCAGC	Screening of LCABL_27650 mutants (RR14)
C96-50721	GTCACCTTCTCTAAGGCTGC	Screening of *rrp2* mutants (RR15)
C91-15689	CCTGCATATAAGAACCCC	Screening of *rrp3* mutants (RR16)

Table S4. Maximum specific growth rates and ΔOD_{595} of different *L. casei* BL23 mutants grown in MRS broth in microplates.

Strain	Maximum specific growth rate, μ_{max} (h^{-1})	OD_{max}-OD_{min}
BL23	0.279 ± 0.005	2.422 ± 0.06
TC01	0.271 ± 0.006	2.344 ± 0.14
TC02	0.291 ± 0.012	2.224 ± 0.08
TC03	0.284 ± 0.005	2.285 ± 0.11
TC04	0.239 ± 0.007 (P<0.01)[a]	2.390 ± 0.09
TC05	0.268 ± 0.015	2.386 ± 0.06
TC06	0.280 ± 0.006	2.154 ± 0.12
TC07	0.274 ± 0.010	2.316 ± 0.08
TC08	0.275 ± 0.001	2.445 ± 0.09
TC09	0.279 ± 0.015	2.400 ± 0.03
TC10	0.282 ± 0.006	2.382 ± 0.14
TC11	0.213 ± 0.002 (P<0.01)	2.318 ± 0.08
TC12	0.285 ± 0.004	1.883 ± 0.06 (P<0.01)
TC13	0.284 ± 0.010	2.246 ± 0.12
TC14	0.277 ± 0.004	2.377 ± 0.13
TC15	0.266 ± 0.016	2.269 ± 0.18
TC16	0.276 ± 0.005	2.249 ± 0.16
TC17	0.281 ± 0.014	2.341 ± 0.18

[a] Significant difference (P<0.01; one way ANOVA) between the wild-type and the mutant strain.

Table S5 A. Maximal growth rate (μ_{max}) values and pairwise two way ANOVA under different stress conditions[a].

Strain	0.5% bile			0.6 M NaCl			pH 3.75			T 42°C		
		ANOVA[b]			ANOVA			ANOVA			ANOVA	
	μ_{max}	Strain	Int.[c]	μ_{max}	Strain	Int.	μ_{max}	Strain	Int.	μ_{max}	Strain	Int.
BL23	0.17±<10^{-2}			0.23±0.01			0.07±<10^{-2}			0.26±0.01		
TC01	NG[d]	-[e]	-	NG	-	-	0.03±<10^{-2}	**<0.001**	**<0.001**	0.24±<10^{-2}	<0.001	0.030
TC02	0.17±0.01	0.778	0.010	0.23±0.01	0.097	0.056	0.05±<10^{-2}	0.902	0.005	0.25±0.02	0.725	0.051
TC03	0.17±<10^{-2}	0.848	0.070	0.20±0.01	**0.002**	**<0.001**	0.06±<10^{-2}	0.179	0.003	0.25±0.01	0.368	0.010
TC04	0.13±0.01	<0.001	0.307	0.16±0.01	**<0.001**	**<0.001**	0.05±<10^{-2}	**<0.001**	**<0.001** (-)[f]	0.23±0.01	<0.001	0.033
TC05	0.17±<10^{-2}	0.154	0.201	0.22±0.02	0.037	0.560	0.05±0.01	0.002	0.289	0.24±0.01	0.006	0.444
TC06	NG	-	-	NG	-	-	0.03±0.01	**<0.001**	**<0.001**	0.17±0.01	**<0.001**	**<0.001**
TC07	0.18±0.01	0.472	0.459	0.23±<10^{-2}	0.077	0.743	0.04±0.01	**<0.001**	**0.009**	0.26±0.01	0.452	0.511
TC08	0.19±0.01	0.153	0.009	0.23±0.01	0.163	0.911	0.07±<10^{-2}	0.439	0.005	0.25±<10^{-2}	0.007	0.333
TC09	0.18±<10^{-2}	0.557	0.556	0.22±<10^{-2}	0.209	0.205	0.07±<10^{-2}	0.049	0.062	0.26±0.01	0.735	0.726
TC10	0.15±<10^{-2}	**<0.001**	**<0.001**	0.22±0.01	0.135	0.019	0.05±<10^{-2}	0.014	0.002	0.24±0.01	**0.003**	**<0.001**
TC11	0.13±<10^{-2}	**<0.001**	**0.001** (-)	0.17±0.01	<0.001	0.591	0.05±<10^{-2}	**<0.001**	**<0.001** (-)	0.20±0.01	<0.001	0.110
TC12	0.08±<10^{-2}	**<0.001**	**<0.001**	0.21±0.01	**0.009**	**<0.001**	NG	-	-	0.22±0.02	**0.005**	**<0.001**
TC13	0.17±0.01	0.971	0.135	0.25±0.01	0.031	0.260	0.04±<10^{-2}	**0.001**	**0.008**	0.24±0.01	0.017	0.001
TC14	0.19±0.01	0.108	0.037	0.22±0.01	0.047	0.144	0.05±<10^{-2}	**0.001**	**0.006**	0.24±0.01	0.007	0.020
TC15	0.16±0.01	0.002	0.559	0.22±0.01	0.012	0.828	0.04±<10^{-2}	0.025	0.487	0.24±0.01	0.003	0.515
TC16	0.17±0.01	0.162	0.624	0.21±0.02	0.015	0.046	0.05±<10^{-2}	**<0.001**	**0.004**	0.24±0.01	0.043	0.166
TC17	0.16±0.01	0.252	0.123	0.20±0.02	0.015	0.008	0.06±<10^{-2}	0.254	0.063	0.24±0.01	0.092	0.042

[a] Contribution of treatment is omitted since it was always significant; significant differences are indicated in bold characters.
[b] P values
[c] Interaction.
[d] No growth.
[e] Analysis not performed.
[f] Negative interaction.

Table S5 B. Increment in optical density (Δ O.D.) values and pairwise two way ANOVA at different stress conditions[a].

Strain	0.5% bile			0.6 M NaCl			pH 3.75			T 42°C		
		ANOVA			ANOVA			ANOVA			ANOVA	
	Δ O.D.	Strain	Int.	Δ O.D.	Strain	Int.	Δ O.D.	Strain	Int.	Δ O.D.	Strain	Int.
BL23	1.15±0.14			1.83±0.04			0.56±0.07			2.19±0.10		
TC01	NG	-	-	NG	-	-	0.19±0.06	**<0.001**	**<0.005**	2.07±0.15	0.112	0.741
TC02	1.16±0.10	0.061	0.046	1.86±0.05	0.010	0.003	0.40±0.02	<0.001	0.569	1.97±0.08	<0.001	0.848
TC03	1.13±0.03	0.114	0.240	1.75±0.05	0.003	0.419	0.35±0.02	<0.001	0.337	2.11±0.17	0.072	0.591
TC04	0.85±0.07	0.004	0.014	1.81±0.02	0.404	0.873	0.38±0.04	0.006	0.043	2.18±0.07	0.653	0.751
TC05	1.21±0.09	0.781	0.317	1.88±0.07	0.911	0.174	0.37±0.04	0.001	0.015	2.15±0.15	0.420	0.962
TC06	NG	-	-	NG	-	-	0.14±0.01	**<0.001**	**0.009**	0.46±0.03	**<0.001**	**<0.001**
TC07	1.11±0.07	0.126	0.495	1.84±0.01	0.128	0.086	0.40±0.02	0.001	0.037	2.02±0.11	0.008	0.489
TC08	1.11±0.05	0.820	0.484	1.88±0.08	0.304	0.725	0.41±0.02	0.086	0.025	2.10±0.12	0.409	0.192
TC09	1.23±0.10	0.632	0.375	1.91±0.03	0.278	0.056	0.40±0.02	0.007	0.027	2.12±0.10	0.287	0.579
TC10	0.98±0.03	0.070	0.245	1.82±0.03	0.516	0.685	0.36±0.04	0.022	0.111	2.14±0.14	0.425	0.927
TC11	1.09±0.11	0.135	0.730	1.73±0.11	0.023	0.966	0.32±0.02	<0.001	0.071	2.11±0.12	0.082	0.805
TC12	0.52±0.03	<0.001	0.297	1.81±0.03	**<0.001**	**<0.001** (-)	NG	-	-	1.02±0.05	**<0.001**	**<0.001**
TC13	1.15±0.05	0.132	0.138	1.89±0.03	0.114	-0.008	0.34±0.01	<0.001	0.622	2.16±0.08	0.054	0.156
TC14	1.13±0.02	0.549	0.847	1.79±0.06	0.316	0.930	0.45±0.04	0.097	0.477	2.14±0.09	0.359	0.991
TC15	1.16±0.08	0.261	0.220	1.89±0.02	0.328	-0.044	0.49±0.09	0.042	0.413	2.15±0.09	0.119	0.348
TC16	1.19±0.06	0.266	0.105	1.92±0.03	0.322	0.012	0.48±0.02	0.018	0.341	2.09±0.09	0.027	0.528
TC17	1.15±0.03	0.489	0.485	1.72±0.05	0.072	0.773	0.58±0.04	0.605	0.356	2.01±0.11	0.054	0.434

[a] Abbreviations and symbols as in Table 3 A.

Table S6. MIC values of *L. casei* BL23 and TCS-defective derivative mutants.

Strain	Bacitracin[a]	Vancomycin[b]	Gramicidin[a]	Nisin[a]
BL23	3	0.97[c]	>250	1
TC01	1	0.19[c]	>250	0.25
TC02	3	>1.2	>250	1
TC03	3	>1.2	>250	1
TC04	4	>1.2	>250	1.5
TC05	3	>1.2	>250	1
TC06	3	0.45[c]	>250	0.25
TC07	3	>1.2	>250	1
TC08	3	>1.2	>250	1
TC09	2	>1.2	>250	0.25
TC10	2	>1.2	>250	0.25
TC11	3	>1.2	>250	0.5
TC12	3	0.17[c]	>250	0.25
TC13	3	>1.2	>250	1
TC14	3	>1.2	>250	1
TC15	4	>1.2	>250	1
TC16	4	>1.2	>250	1
TC17	4	>1.2	>250	1
ΔRR01	1.5	0.29[c]	>250	0.25
ΔRR01-c	3	0.74[c]	>250	1
ΔRR06	3	0.43[c]	>250	0.5
ΔRR06-c	3	0.97[c]	>250	1
ΔRR12	3	0.45[c]	>250	0.125

[a] µg ml^{-1}

[b] mg ml^{-1}

[c] IC_{50} values are shown. MIC values higher than 1.2 mg ml^{-1}.

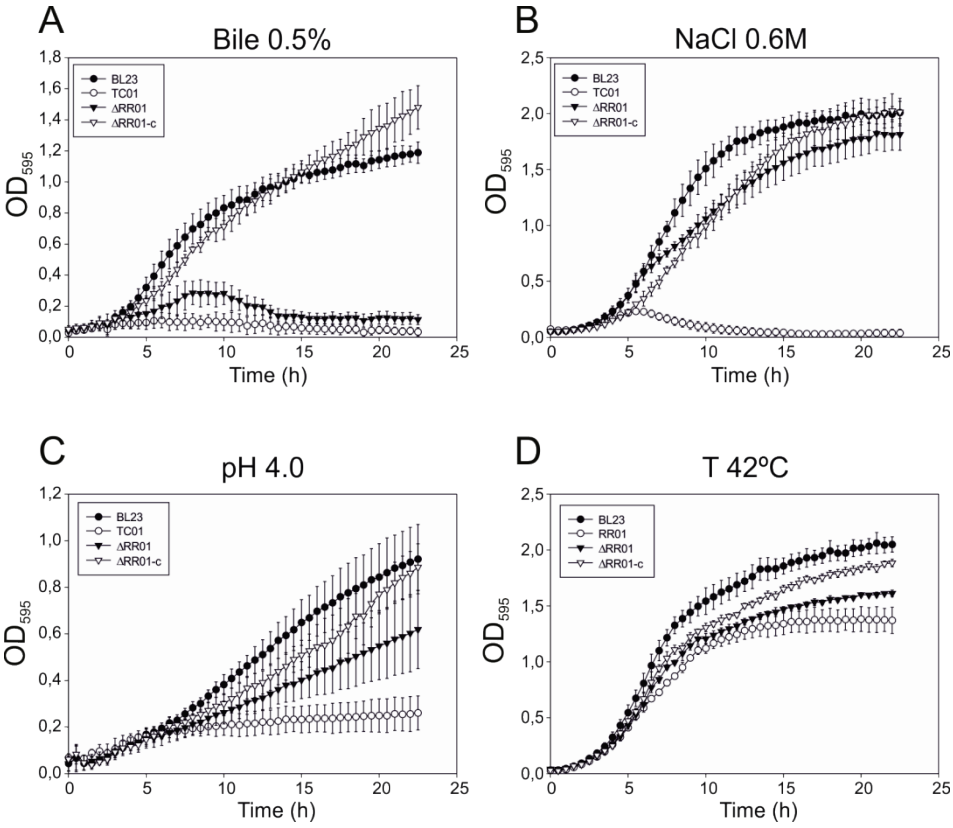


Figure S1. Growth of BL23 and TC01-defective mutants under different conditions (TC01, insertional mutant; ΔRR01, deletion mutant; ΔRR01-c, deletion mutant complemented with plasmid pT1-RR01). Error bars indicate SD (at least three replicates).

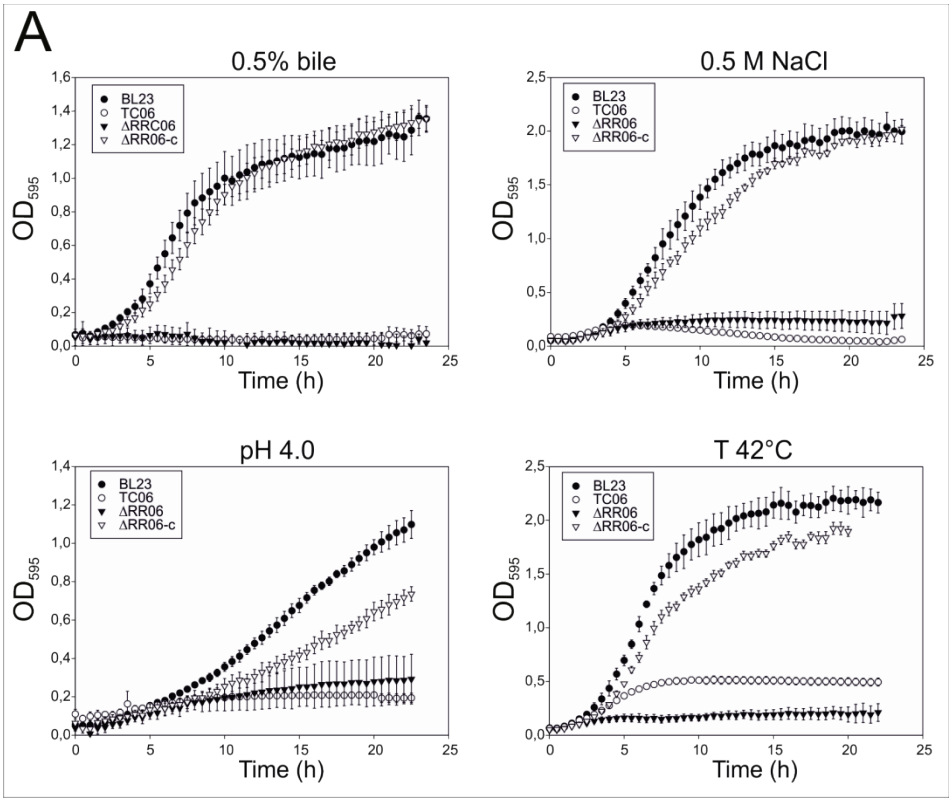


Figure S2 A. Growth of BL23 and TCS06-defective mutants under different conditions. Error bars indicate SD (at least three replicates).

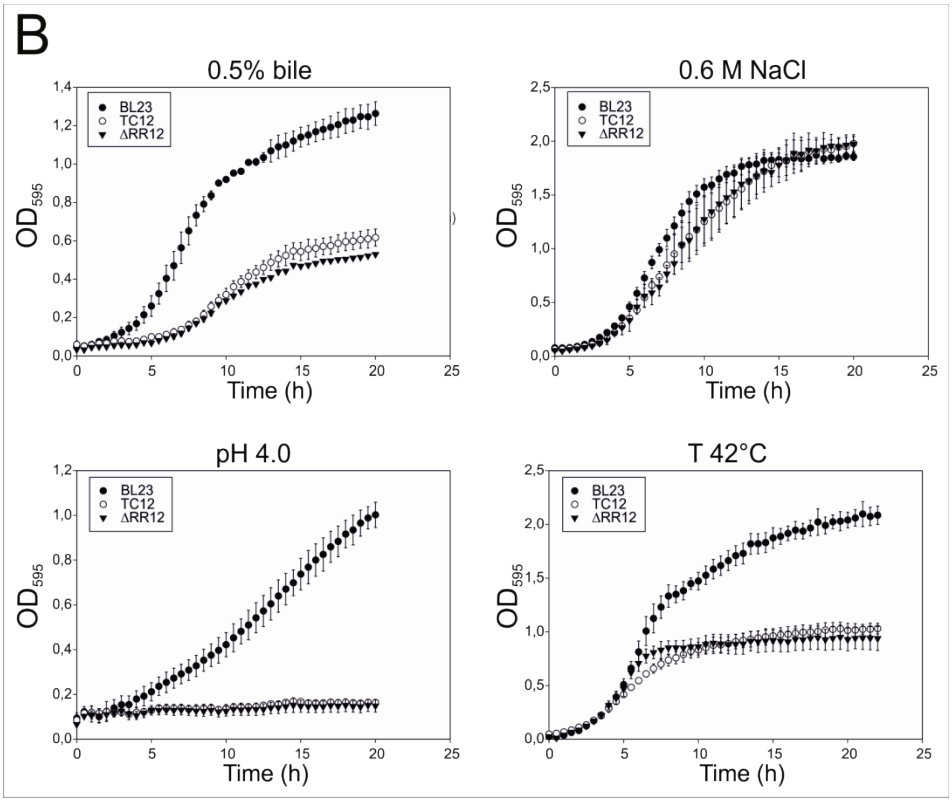


Figure S2 B. Growth of BL23 and TCS12-defective mutants under different conditions. Error bars indicate SD (at least three replicates).

REFERENCES

Azcarate-Peril, M. A., O. McAuliffe*, et al.* (2005). "Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in Lactobacillus acidophilus." Appl. Environ. Microbiol. **71**(10): 5794-5804.

Comenge, Y., R. Quintiliani, Jr*., et al.* (2003). "The CroRS two-component regulatory system is required for intrinsic beta-lactam resistance in *Enterococcus faecalis*." J Bacteriol **185**(24): 7184-7192.

Corcoran, B. M., C. Stanton*, et al.* (2008). "Life under stress: the probiotic stress response and how it may be manipulated." Curr. Pharm. Des. **14**(14): 1382-1399.

de Vrese, M. and J. Schrezenmeir (2008). Probiotics, prebiotics, and synbiotics. Adv. Biochem. Eng. Biotechnol. **111:** 1-66.

Fujii, T., C. Ingham*, et al.* (2008). "Two homologous Agr-like quorum-sensing systems cooperatively control adherence, cell morphology, and cell viability properties in *Lactobacillus plantarum* WCFS1." J Bacteriol **190**(23): 7655-7665.

Hancock, L. E. and M. Perego (2004). "Systematic inactivation and phenotypic characterization of two-component signal transduction systems of *Enterococcus faecalis* V583." J. Bacteriol. **186**(23): 7951-7958.

Hazen, T. C. and D. A. Stahl (2006). "Using the stress response to monitor process control: pathways to more effective bioremediation." Curr Opin Biotechnol **17**(3): 285-290.

Hobbs, E. C., J. L. Astarita*, et al.* (2010). "Small RNAs and small proteins involved in resistance to cell envelope stress and acid shock in *Escherichia coli*: analysis of a bar-coded mutant collection." J Bacteriol **192**(1): 59-67.

Jordan, S., M. I. Hutchings*, et al.* (2008). "Cell envelope stress response in Gram-positive bacteria." FEMS Microbiology Reviews **32**(1): 107-146.

Kobayashi, K., M. Ogura*, et al.* (2001). "Comprehensive DNA microarray analysis of *Bacillus subtilis* two-component regulatory systems." J Bacteriol **183**(24): 7365-7370.

Landete, J. M., L. García-Haro*, et al.* (2010). "Requirement of the *Lactobacillus casei* MaeKR two-component system for L-malic acid utilization via a malic enzyme pathway." Appl. Environ. Microbiol. **76**(1): 84-95.

Le Breton, Y., G. Boël*, et al.* (2003). "Molecular characterization of *Enterococcus faecalis* two-component signal transduction pathways related to environmental stresses." Environ Microbiol **5**(5): 329-337.

Le Breton, Y., C. Muller*, et al.* (2007). "New Insights into the *Enterococcus faecalis* CroRS Two-Component System Obtained Using a Differential-Display Random Arbitrarily Primed PCR Approach." Appl Environ Microbiol **73**(11): 3738-3741.

Leloup, L., S. D. Ehrlich*, et al.* (1997). "Single-crossover integration in the *Lactobacillus sake* chromosome and insertional inactivation of the *ptsI* and *lacL* genes." Appl. Environ. Microbiol. **63**(6): 2117-2123.

Makarova, K., A. Slesarev*, et al.* (2006). "Comparative genomics of the lactic acid bacteria." Proc Natl Acad Sci U S A **103**(42): 15611-15616.

Maldonado-Barragan, A., J. L. Ruiz-Barba*, et al.* (2009). "Knockout of three-component regulatory systems reveals that the apparently constitutive plantaricin-production phenotype shown by *Lactobacillus plantarum* on solid medium is regulated via quorum sensing." Int J Food Microbiol **130**(1): 35-42.

Mazé, A., G. Boël*, et al.* (2010). "Complete Genome Sequence of the Probiotic *Lactobacillus casei* Strain BL23." J Bacteriol **192**(10): 2647-2648.

Morel-Deville, F., F. Fauvel*, et al.* (1998). "Two-component signal-transducing systems involved in stress responses and vancomycin susceptibility in *Lactobacillus sakei*." Microbiology **144 (Pt 10)**: 2873-2883.

Muller, C., M. Sanguinetti*, et al.* (2008). "Characterization of two signal transduction systems involved in intracellular macrophage survival and environmental stress response in *Enterococcus faecalis*." J Mol Microbiol Biotechnol **14**(1-3): 59-66.

Pfeiler, E. A., M. A. Azcarate-Peril*, et al.* (2007). "Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*." J Bacteriol **189**(13): 4624-4634.

Posno, M., R. J. Leer*, et al.* (1991). "Incompatibility of *Lactobacillus* Vectors with Replicons Derived from Small Cryptic *Lactobacillus* Plasmids and Segregational Instability of the Introduced Vectors." Appl. Environ. Microbiol. **57**(6): 1822-1828.

Risoen, P. A., L. S. Havarstein*, et al.* (1998). "Identification of the DNA-binding sites for two response regulators involved in control of bacteriocin synthesis in *Lactobacillus plantarum* C11." Mol Gen Genet **259**(2): 224-232.

Santivarangkna, C., U. Kulozik*, et al.* (2008). "Inactivation mechanisms of lactic acid starter cultures preserved by drying processes." J Appl Microbiol **105**(1): 1-13.

Schotte, L., L. Steidler*, et al.* (2000). "Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*." Enzyme Microb Technol **27**(10): 761-765.

Stock, A. M., V. L. Robinson*, et al.* (2000). "Two-component signal transduction." Annu Rev Biochem **69**: 183-215.

Sturme, M. H., C. Francke*, et al.* (2007). "Making sense of quorum sensing in lactobacilli: a special focus on *Lactobacillus plantarum* WCFS1." Microbiology **153**(Pt 12): 3939-3947.

Winkler, M. E. and J. A. Hoch (2008). "Essentiality, bypass, and targeting of the YycFG (VicRK) two-component regulatory system in gram-positive bacteria." J Bacteriol **190**(8): 2645-2648.

CHAPTER 1

PART II

Influence on the general physiology of *Lactobacillus casei* BL23 of Two-Component Signal Transduction Systems

Ainhoa Revilla-Guarinos, Cristina Alcántara Baena and Manuel Zúñiga

Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC) PO Box 73, 46100 Burjassot, Valencia, Spain

ABSTRACT

Lactobacillus casei is a lactic acid bacterium commonly found in the gastrointestinal tract of animals, in plant materials and in dairy products. The ability of *L. casei* to colonize such a wide range of environments suggests that it possesses efficient systems of sensing and signal transfer that let this organism adapt its physiology to different environmental conditions. *L. casei* BL23 harbours 17 two-component signal transduction systems (TCS) (Alcántara *et al.*, 2011). With the aim of determining the role of these TCS in *L. casei* physiology, growth of 17 *L. casei* BL23 derivative strains defective in each TCS was monitored under different conditions. Inactivation of systems TCS04 (LCABL_10480-LCABL_10490), TCS11 (LCABL_18970-LCABL_18980) and TCS12 (LCABL_19600-LCABL_19610) resulted in growth defects under reference conditions (growth in MRS medium at 37°C without shaking) (Alcántara *et al.*, 2011). This study deepens in the involvement of TCS in the physiology of *L. casei* BL23. Additional assays showed that TCS11 might be controlling functions related with the ability of BL23 to growth in milk. On the other side, TCS12 might be controlling properties of the cell surface that would account for its higher sensitivity to low pH and altered morphology.

INTRODUCTION.

Lactobacillus casei is a facultative heterofermentative lactic acid bacterium of interest for food industry as a starter culture for milk fermentation and for maturation of some types of cheeses. During fermentation, growth of *L. casei* is accompanied by lactic acid production, leading to acidification of the growing media. Previous studies of our group had shown that *L. casei* BL23 harbours 17 putative TCS (Alcántara *et al.*, 2011). Insertional mutants were obtained for each RR indicating that none of the TCS is essential for growth (Alcántara *et al.*, 2011). BL23 derivative strain harboring a complete deletion of gene LCABL_19600 (RR12) was also obtained. Complementation of ΔRR12 was not achieved because this strain resulted impervious to transformation; notwithstanding, the similarity of the phenotypes of strains TC12 (insertional mutant) and ΔRR12 suggested that the observed effects were mainly due to inactivation of RR12 (Alcántara *et al.*, 2011).

The growth of *L. casei BL23* and their derivative RR-defective mutants was compared in reference conditions (MRS at 37 °C without shaking) and stress conditions (Alcántara *et al.*, 2011). The conditions assayed were: MRS supplemented with 0.5% bile, MRS supplemented with 0.6 M NaCl, MRS adjusted to pH 3.75 and growth in MRS at 42 °C. Results showed that the growth rates (μ_{max}) of the different mutants were similar to that of the wild type strain in reference conditions excepting TC04 and TC11 mutants which displayed significantly reduced growth rates relative to the wild type. On the other hand, the maximum cell density of TC12 (ΔOD_{595}) was significantly lower than that of the wild-type strain. Furthermore, the inactivation of TCS01, TCS06 and TCS12 led to major growth defects under stress conditions (Alcántara *et al.*, 2011). The growth of the three mutants was impaired in the presence of 0.5 % bile and acidic pH. Mutant TC06 was not able to growth in the presence of 0.6 M NaCl. Temperature affected the growth of mutants TC06 and TC12 (Alcántara *et al.*, 2011).

The effect of the inactivation of *L. casei* TCS on the response against antibiotics targeted to the cell envelope was also assayed (Alcántara *et al.*, 2011). Inactivation of ten out of 17 TCS (mutants TC01, TC04, TC06, TC09,

TC10, TC11, TC12, TC15, TC16 and TC17) led to differences in antibiotic sensitivities relative to the wild type (Alcántara *et al.*, 2011).

The objectives of the present study were, firstly to further characterize the involvement of TCS of *L. casei* BL23 in its general physiology by studying the performance of the different RR mutants when growing in skim milk; secondly, to obtain more information about the specific role of TCS12.

MATERIALS AND METHODS.

Bacterial strains and growth conditions.

The strains used in this study are listed in Table 1. *L. casei* strains were routinely grown in MRS broth (Oxoid) at 37 °C under static conditions. Erythromycin 5 µg ml^{-1} was added when required. The corresponding solid media were prepared by adding 1.5% agar. Strains were stored at -80 °C in their corresponding growth media supplemented 20% glycerol (v/v).

Growth in skim milk

For assays of growth in milk, cells were grown in MRS broth without glucose and supplemented with 0.5% lactose for 24 h at 37°C, centrifuged, washed twice with one volume of 0.1% (w/v) peptone water and resuspended in distilled water. The optical density at 595nm (OD_{595}) of the cell suspension was adjusted to 10.0 and 1 ml of cell suspension was added to 50 ml of reconstituted skim milk (10% w/v). The cultures were incubated at 37°C without stirring. Variation of pH was monitored and after 40 hours viable cell countings were determined in MRS agar plates.

Phenotypic characterization of TCS12 mutant strains

The growth of BL23 and TC12 strains in buffered media was studied. The cultures were prepared as follows: cells from frozen stocks were inoculated in MRS agar plates and incubated at 37 °C. Cells from single colonies were inoculated in MRS medium and grown at 37 °C for 16 h (stationary phase). Subsequently, the cultures were centrifuged, washed twice with one volume of 0.1 % (w/v) peptone water, suspended in peptone water and inoculated to a final OD_{595} of 0.05 in 250 µl of MRS media adjusted to pH 6.75 with 0.1 M or 0.2 M potassium phosphate buffer that were subsequently dispensed in 96 well microtiter plates. Growth was monitored for 24 hours at 37 °C by changes in the OD_{595} in a multidetection microplate reader from BMG Labtech. The pH of the cultures media and viable cell countings (cfu ml^{-1}) at the end of growth were determined.

Table 1. Bacterial strains used in this study.

Strain	Relevant characteristics [a]	Ref. or source
L. casei BL23	Wild type	B. Chassy, U. Illinois
L. casei TC01	LCABL_02080 mutant; pRV02080 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC02	LCABL_05260 mutant; pRV05260 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC03	LCABL_07760 mutant; pRV07760 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC04	LCABL_10480 mutant; pRV10480 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC05	LCABL_10640 mutant; pRV10640 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC06	LCABL_12050 mutant; pRV12050 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC07	LCABL_14280 mutant; pRV14280 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC08	LCABL_14430 mutant; pRV14430Ery^r	(Alcántara *et al.*, 2011)
L. casei TC09	LCABL_16430 mutant; pRV16430 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC10	LCABL_18830 mutant; pRV18830 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC11	LCABL_18980 mutant; pRV18980 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC12	LCABL_19600 mutant; pRV19600 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC13	LCABL_25620 mutant; pRV25620 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC14	LCABL_27650 mutant; pRV27650 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC15	LCABL_28720 mutant; pRV28720 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC16	LCABL_30130 mutant; pRV30130 Ery^r	(Alcántara *et al.*, 2011)
L. casei TC17	BL23 Δ*maeR*	BL315 (Landete *et al.*, 2010)
L. casei ΔRR12	BL23 Δ*rrp1* (LCABL_19600)	(Alcántara *et al.*, 2011)

[a] Ery^r, erythromycin resistance.

The ability to maintain the pH homeostasis during growth of *L. casei* BL23 and derivative strain ΔRR12 was studied. Cells were exposed to media adjusted at different acidic pH values and their ability to recover growth was monitored by changes in OD_{595}. The strains were inoculated in MRS to a final OD_{595} of 0.05 and incubated at 37 °C (reference condition) to mid-exponential phase (OD_{595} 0.5-0.6). At this point, 3 samples of 1 ml from each culture were collected, washed with one volume of 0.1% peptone water, suspended in MRS (control sample), MRS adjusted to pH 3.5 with HCl or MRS adjusted to pH 3 with HCl and incubated for 1 hour at 37 °C. To determine the effect of the acid exposition on cell viability, serial dilutions of the cultures were performed before and immediately after incubation in acid media and plated in MRS plates for colony counting. Survival was determined by comparing viable cell countings before and after the acid exposure. The remaining cells were collected and washed as previously described. Subsequently, they were suspended in one volume of fresh MRS medium and 250 µl aliquots of each cell suspension were dispensed in 96 wells microtiter plates. Growth was monitored for 20 h by changes in OD_{595} in a microplate reader. The experiment was performed with three independent cultures of each strain.

Microscopic characterization.

Stationary phase cultures of strains BL23 and ΔRR12 grown under reference conditions and in 0.1 M potassium phosphate buffered media where photographed. The cells were observed in a Nikon Eclipse 90i optical microscope with phase contrast objective at 100x magnification. Pictures were made with a refrigerated Digital Sight DS-5Mc camera. The cell sizes, the cell-chains length and the number of cells per chain (n = 100) were determined using the tools implemented in the NIS-Elements software (Nikon). Significant differences ($P<0.01$) between the wild-type and the mutant strain were determined by one-way ANOVA.

RESULTS.

Growth in skim milk

L. casei is used for the production of a number of dairy fermented products. It is then of interest to determine the effect of the inactivation of TCS on the growth of *L. casei* in milk. Results obtained are shown in Figure 1. All strains were able to grow in skim milk and reached similar cfu after 48 h of incubation (Figure 1A). Furthermore, all strains lowered the pH of the medium at a similar rate excepting strain TC11 (Figure 1B) which showed a higher acidification rate.

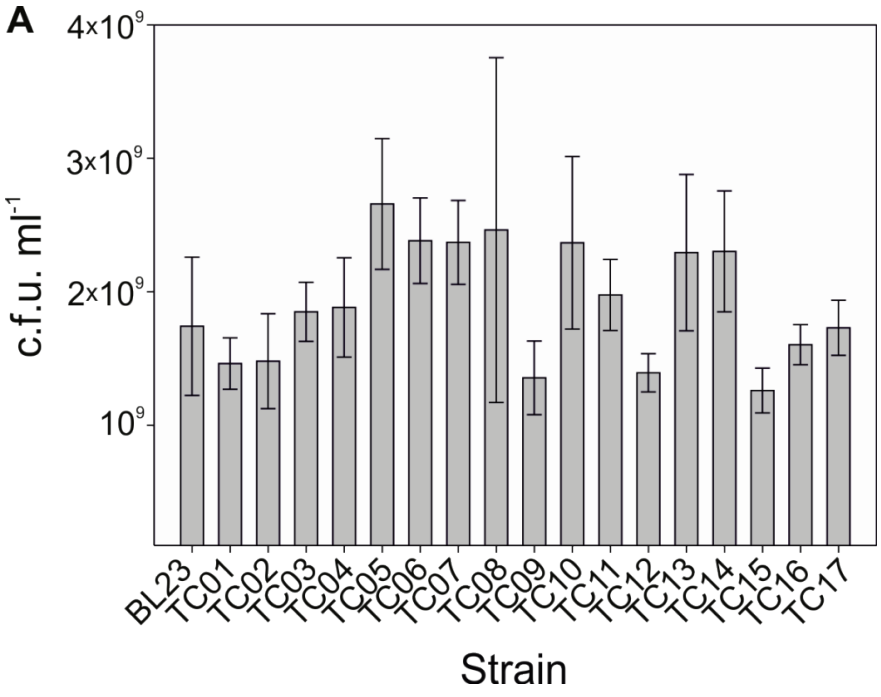


Figure 1A. Growth in skim milk. Viable cell counting of *L. casei* BL23 and derivative mutants after 48 h of growth in skim milk. Error bars indicate SD (four replicates).

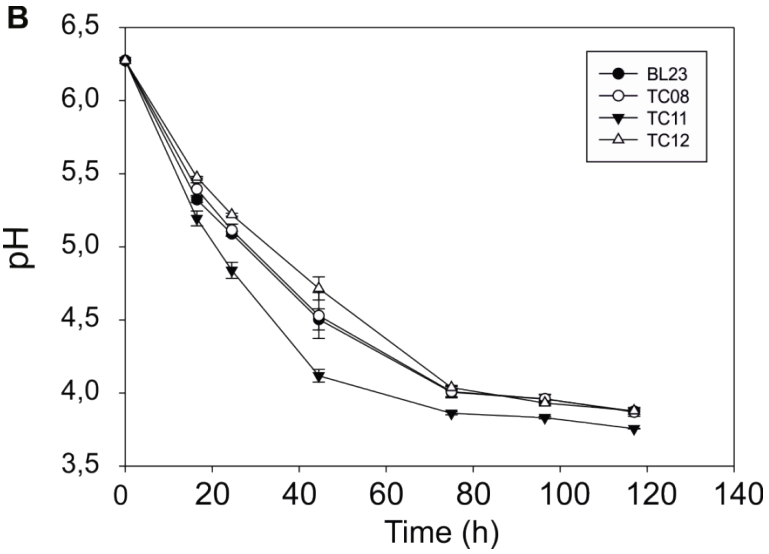


Figure 1B. Growth in skim milk. pH variation during growth in skim milk (only strain BL23 and selected mutants are shown). Error bars indicate SD (four replicates).

Growth of TC12 strain in buffered media

Since mutant TC12 was very sensitive to low pH (Alcántara *et al.*, 2011), an additional assay was devised to determine whether the premature arrest of growth observed for mutant TC12 in reference conditions was due to the acidification of the medium. To this end, the growth of strains BL23 and TC12 was compared in MRS supplemented with potassium phosphate (0.1 and 0.2 M). Growth was monitored and viable cell countings (cfu) and pH were determined at the end of growth.

Addition of potassium phosphate resulted in an increase of ΔOD_{595} in TC12 which reached values similar to those observed for BL23 (Figure 2, panels A and B). Furthermore, BL23 reached higher cfu values than TC12 mutant in all concentrations of potassium phosphate assayed but the differences between both strains were greatly diminished in medium containing 0.1 or 0.2 M potassium phosphate (Figure 2, panel C). In addition, significant differences in the final pH value of the culture medium were only observed in MRS ($P < 0.001$; Figure 2, panel C). These results indicate that the decrease of pH during

growth explains the lower OD_{595} reached by strain TC12 in reference conditions.

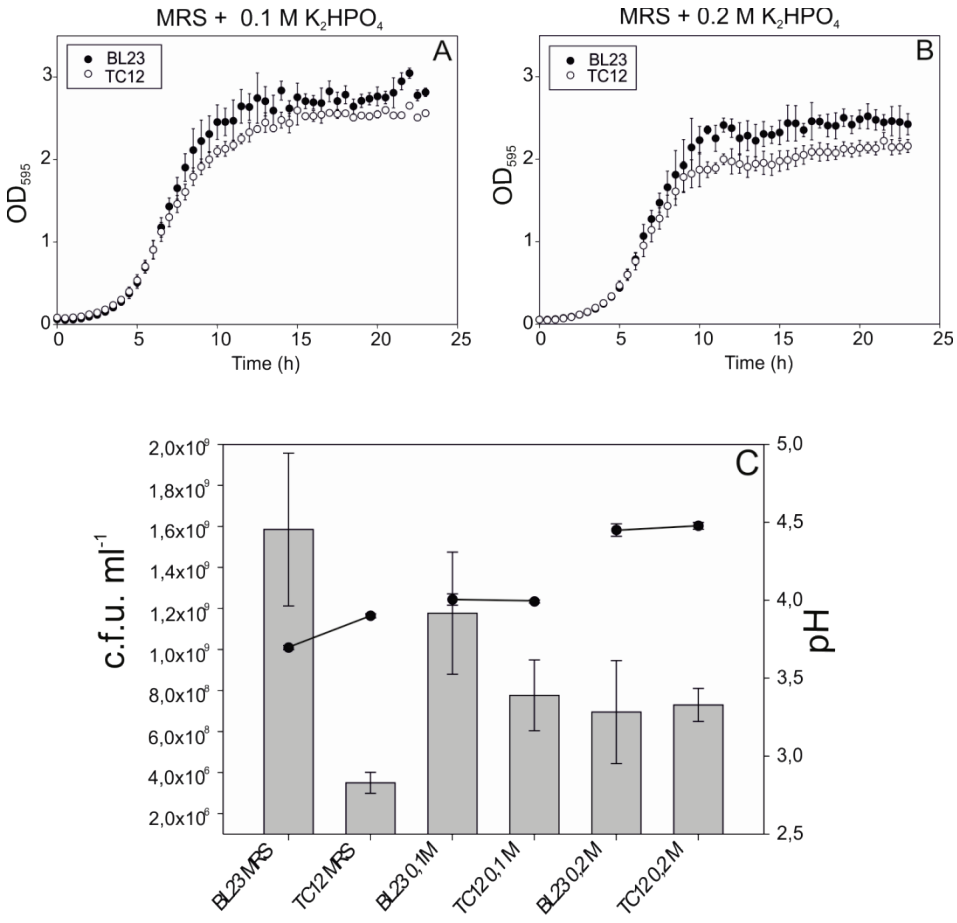


Figure 2. Effect of buffered media in growth. Growth of BL23 and mutant TC12 in MRS supplemented with 0.1 M (A) or 0.2 M (B) potassium phosphate. (C) Viable cell counting (grey bars) and final pH (black dots) determined after 24 h of growth in reference conditions and buffered media. Error bars indicate SD (six replicates).

pH homeostasis studies with BL23 and ΔRR12 strains.

We decided to investigate if the premature arrest of growth of ΔRR12 in reference conditions (Alcántara *et al.*, 2011) could be related with an impaired ability to regulate pH homeostasis. *L. casei* BL23 and derivative strain ΔRR12 were grown in reference conditions to mid-exponential phase. At this point, cells were collected and exposed to acidic media for one hour (pH 3 and 3.5). Control cells were exposed to MRS for the same time. Subsequently, they were transferred to reference conditions and growth was monitored.

In both strains, control cells exposed for one hour to MRS continued growing when put back to reference conditions (Figure 3, A and B, white circles). The results showed that after one hour of exposition to pH 3.5 and pH 3 the wild-type strain cells were able to immediately resume growth upon putting them back to reference conditions (Figure 3A). ΔRR12 strain behaved like the wild type after exposure to pH 3.5 (Figure 3B). However after being exposed to pH 3 for one hour, ΔRR12 only resumed growth after 15 hours of latency (data not shown).

Viability results (Figure 3C) after one hour of exposition to MRS (control cells) showed a survival rate between 150 and 200 % for both strains, indicating that growth continued as expected. Exposition to pH 3.5 for one hour had a bacteriostatic effect on both strains since the survival rate after 1 hour at pH 3.5 for the two strains was around 100%. These results suggest that although both strains were able to survive to the acid exposition at pH 3.5, cell division was arrested; this correlated with the growth curves since both strains quickly recovered growth when they were put back to reference conditions (Figure 3, A and B, full square). Exposition to pH 3 for one hour resulted in a 50% loss of viability of BL23 culture and in a 99.998% cell death of the ΔRR12 culture (Figure 3C). This is in agreement with the previous result showing a delay of 15 h before growth was appreciated when ΔRR12 cells incubated at pH 3.0 were put back in fresh MRS (data not shown).

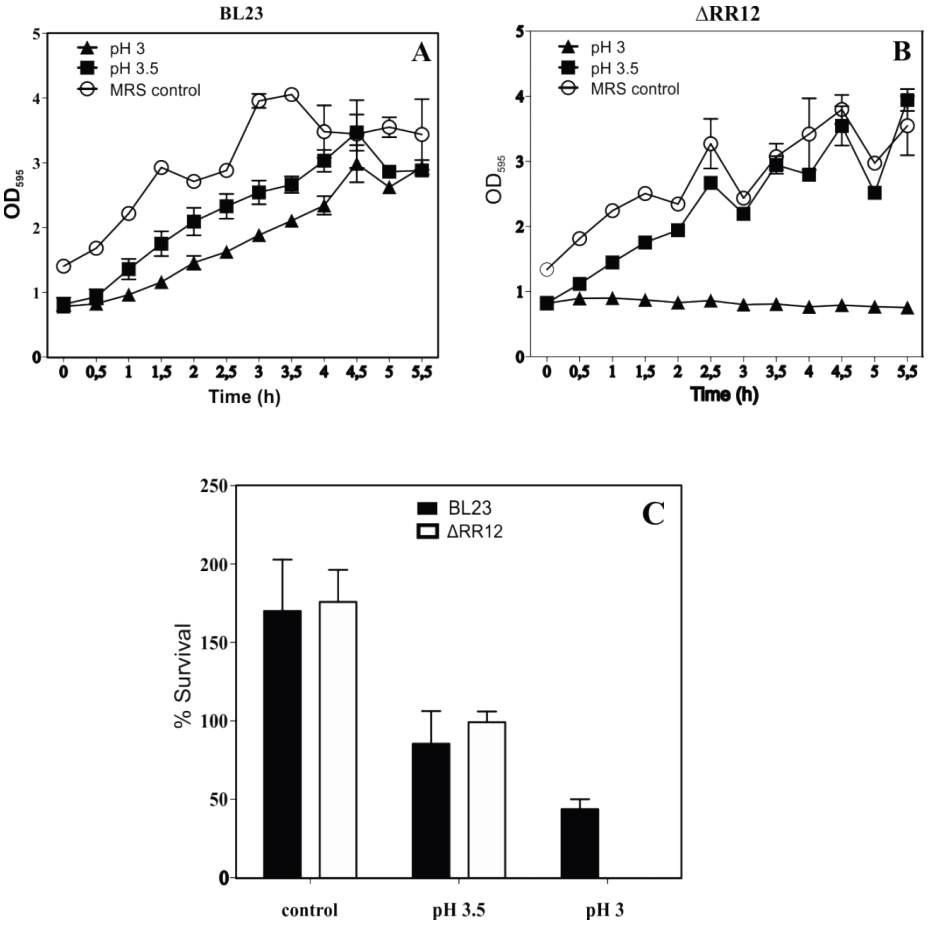


Figure 3. pH homeostasis studies. (A) *L. casei* BL23 and **(B)** ΔRR12, recovery of growth in reference conditions upon one hour of acid exposure. (○) Recovery of control cells exposed to MRS. Recovery of acid challenge cells exposed to MRS adjusted to pH 3.5 (■) and 3 (▲) with HCl. **(C)** Survival rate after the acid challenge (indicated in the x-axis).

Microscopic characterization of ΔRR12 strain

Microscopic observation of stationary phase cultures of BL23 and ΔRR12 strains grown in reference conditions showed altered morphologies in the mutant strain, longer cells and higher numbers of cells per chain in the mutant strain relative to the wild type (Table 2 and Figure 4A).

In order to determine if these differences were caused by the acidification of the media in *Lactobacillus* growing cultures, a new experiment was devised. Stationary phase cultures of BL23 and ΔRR12 grown in MRS supplemented with 0.1 M potassium phosphate buffer were photographed and evolution of pH and OD_{595} during growth was followed.

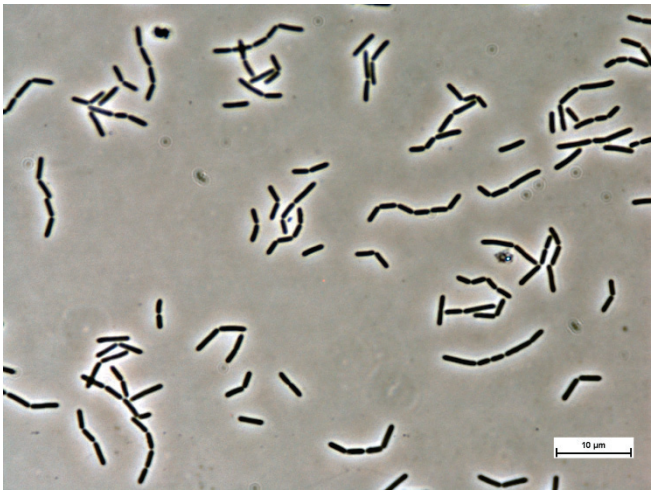
pH values decreased similarly in both strains in both conditions (final pH 3.9 in MRS and 4.1 in MRS 0.1 M potassium phosphate, for both strains). Both strains reached the same final OD_{595} value in buffered media (6.21) that was higher than in reference conditions (5.7 for BL23 and 5.01 for ΔRR12). However, ΔRR12 altered morphologies where detected in both conditions (Figure 4). Significant differences ($P<0.01$) in cell length in buffered media, as well as cell-chain length in both conditions, between the wild type and the mutant strain were found (Table 2). There is a 22% decrease of cellular size and 18% decrease in the number of cells per chain in BL23 grown in buffered media relative to reference conditions. However, ΔRR12 cell size only decreases a 4% and the number of cells per chain a 6% when comparing the same conditions. As a consequence the cell-chains length decreases a 45% for BL23 and only a 17% for ΔRR12 in buffered media relative to reference conditions (Table 2).

Table 2. Morphologic characterization of stationary phase cultures of BL23 and ΔRR12 strains grown in reference conditions (MRS) and in buffered media (MRS + 0.1 M K_2HPO_4).

	Cell length (μm)		Cell-chains length (μm)		Number of cells per chain	
Strain	MRS	MRS +0.1M K_2HPO_4	MRS	MRS +0.1 M K_2HPO_4	MRS	MRS +0.1 M K_2HPO_4
BL23	2,94 ± 0,82	2,29 ± 0,79	7,92 ± 2,55	4,34 ± 1,22	2,6 ± 0,98	2,13 ± 0,35
ΔRR12	3,89 ± 1,62	3,73 ± 6,09*	13,4 ± 9,62*	11,07 ± 7,97*	3,65 ± 2,5	3,42 ± 1,7

*Statistically significant difference ($P<0.01$) relative to the wild type in the same condition.

BL23



ΔRR12

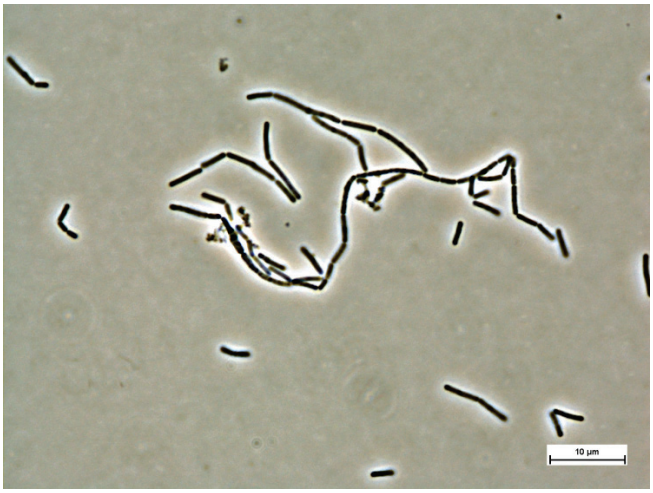
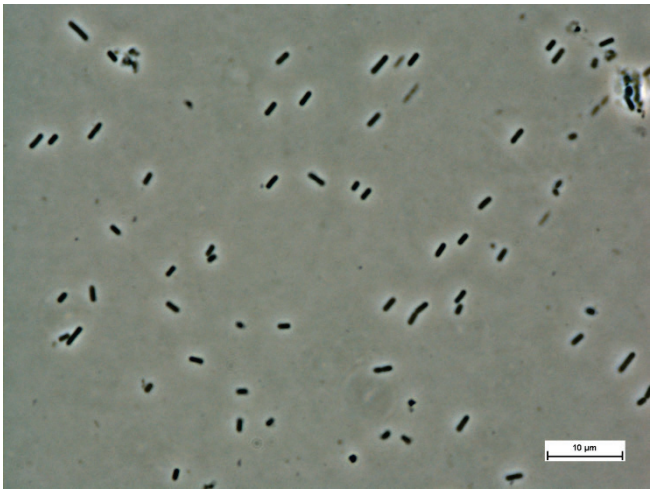


Figure 4 A. Morphologic characterization of stationary phase cultures of BL23 and ΔRR12 strains grown in reference conditions (MRS). Scale bar 10 µm.

BL23



ΔRR12

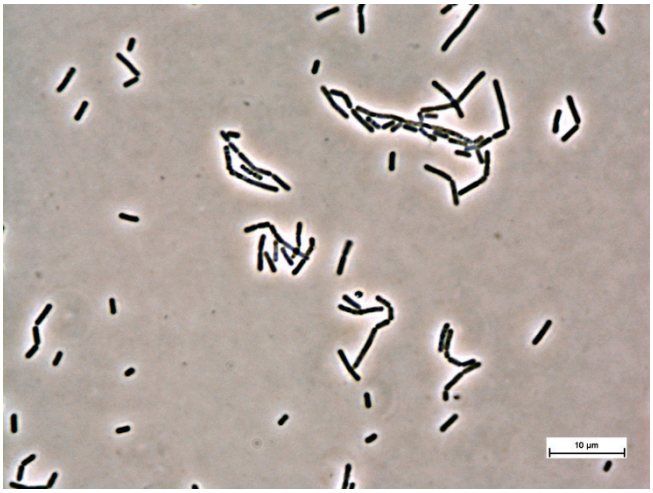


Figure 4 B. Morphologic characterization of stationary phase cultures of BL23 and ΔRR12 strains grown in buffered media (MRS + 0.1 M K_2HPO_4). Scale bar 10 µm.

DISCUSSION

L. casei is a probiotic microorganism naturally associated to mammals and also found in cheese (de Vrese and Schrezenmeir, 2008; Kleerebezem and Vaughan, 2009; Muñoz-Provencio *et al.*, 2009; Bäuerl *et al.*, 2013). In order to survive in these environments *L. casei* must be able to adapt its physiology to widely varying conditions. TCS play a major role in the response to stress conditions in most bacteria (Hancock and Perego, 2004; Azcarate-Peril *et al.*, 2005; Pfeiler *et al.*, 2007; Muller *et al.*, 2008; Kawada-Matsuo *et al.*, 2011; Cui *et al.*, 2012). This work deepens in the role of the 17 TCS present in *L. casei* BL23 (Alcántara *et al.*, 2011) in the physiology of this microorganism.

The performance of the TCS mutants when growing in skim milk was studied. Only TC11 mutant showed differences when grown in skim milk: it showed a higher acidification rate than the wild type strain (Figure 1B). Mutant TC11 had a significantly reduced growth rate in MRS medium compared to the parental stain BL23 although it only showed moderate or no growth defects under the stress conditions assayed in a previous study (Alcántara *et al.*, 2011). Overall, these results suggest that TCS11 plays a relevant role in the regulation of processes that affect cell growth in *L. casei* but not specifically in the stress conditions assayed to date. TCS11 is orthologous to the characterized systems EtaRS (also named as Err10-Ehk10) and LlrA/KinA of *E. faecalis* and *Lactococcus lactis*, respectively. Inactivation of *etaR* in *E.faecalis* OG1RF did not result in a growth defect in Brain Heart broth (Le Breton *et al.*, 2003) but the mutant strain was more sensitive to low pH and more resistant to high temperature than the parental strain (Teng *et al.*, 2002). Le Breton et al. confirmed this phenotype in strain JH2-2 and also observed that the defective mutant strain was more sensitive to NaCl and more resistant to bile (Le Breton *et al.*, 2003). As a difference, the orthologous system LrrA/KinA of *L. lactis* MG1363 resulted in impaired growth, loss of induction of the arginine deiminase pathway but higher resistance to acid conditions (O'Connell-Motherway *et al.*, 2000). Furthermore, a system orthologous to TCS11 has been characterized in *L. acidophilus* (Azcarate-Peril *et al.*, 2005). The inactivation of this system (genes LBA1524-LBA1525) resulted in an acid-sensitive phenotype. In agreement with this, the mutant strain was unable to acidify skimmed milk below pH 5.0. In

contrast, inactivation of TCS11 in *L. casei* also resulted in acid sensitivity but in a higher acidification rate in skim milk. Furthermore, microarray transcriptional analyses suggested that LBA1524-LBA1525 is involved in the regulation of the proteolytic activity of *L. acidophilus*. It is tempting to speculate that TCS11 is also involved in the regulation of the proteolytic activity of *L. casei* but additional evidence is required to settle this point.

Growth assays in buffered MRS showed that the defect of growth of TC12 in reference conditions (Alcántara *et al.*, 2011) was mainly due to pH since differences in growth were greatly diminished in MRS supplemented with 0.1 or 0.2 M potassium phosphate (Figure 2 A and B). Furthermore, viability of the mutant increased in buffered media and final pH values reached by TC12 were also very similar to that of BL23 in buffered media (Figure 2C). One explication could be that the RR12 mutant is impaired in its ability to regulate pH homeostasis. *Lactobacillus* maintain a cytoplasmic pH (pH_{in}) more alkaline than the external pH (pH_{out}), and a pH gradient is generated (ΔpH) and maintained constant (Kashket, 1987). When a critical pH_{in} is reached, the ΔpH cannot be maintained, cellular functions are impaired and this determines the entry in stationary phase (Kashket, 1987; Hutkins, 1993). In *L. casei* a ΔpH of around 1 pH unit is maintained and cells grow until pH_{in} reaches values around 4.4 being the pH_{out} around 3.5 (Kashket, 1987). Studies with an *E. coli* mutant impaired in the Na^+/H^+ antiporter activity and in pH homeostasis above pH 8.3, showed that growth was impaired upon the shift to non-permissive pHs but cessation of growth was not due to cell death (Zilberstein *et al.*, 1984). Furthermore, the recovery of the pH homeostasis (readjustment of the pH_{in}), both in the wild type and the mutant, always preceded resumption of growth (Zilberstein *et al.*, 1982). In the aforementioned work, the authors suggested that the need for pH homeostasis during growth was not due to a general pH sensitivity of cytoplasmic proteins but to a specific-pH sensitive function of the cell responsible for the arrest of growth at not-permissive pHs with retention of viability (Zilberstein *et al.*, 1984). We decided to investigate if the premature arrest of growth of RR12 mutants could be related with an impaired ability to regulate pH homeostasis. *L. casei* BL23 and derivative strain ΔRR12 were grown in reference conditions till mid-exponential phase, exposed to acid challenge and subsequently put back to reference conditions where recovery

of growth was monitored (Figure 3). Growth of mutant ΔRR12 was totally impaired and viability dropped drastically when it was exposed to pH 3 (Figure 3, B and C). Results showed firstly, that the shift between pH 3.5 and 3 appeared to be the limit of the pH homeostasis capacity of *L. casei* BL23 and secondly, that the ability to regulate pH homeostasis was affected in ΔRR12 strain, since pH 3.5 had a bacteriostatic effect in both strains and pH 3 led to a 50 % of cell death in the wild type and almost 100% in ΔRR12. These results suggest that TCS12 could be controlling functions essential in the ability of BL23 to adapt to acid environments, for example involved in surface physiology.

Furthermore, the altered morphology of ΔRR12 mutant strain (Figure 4 and Table 2) both in reference conditions and buffered media also support that some cell envelope functions are possibly regulated by TCS12. *L. casei* mutants affected in cell wall functions and showing different morphologies have been previously described. Mutants with defects in the synthesis of D-alanine esters in LTA in *L. casei* ATCC 7469 showed aberrant cell shape and defective cell separation (Neuhaus *et al.*, 1996). In a different study, inactivation of genes LCABL_00230 and LCABL_02770, encoding two cell-wall hydrolases (p40 and p75) of *L. casei* BL23 also resulted in aberrant cell morphologies (Bäuerl *et al.*, 2010). Further studies will be required to ascertain if TCS12 is controlling functions involved in surface physiology in *L. casei* BL23.

In summary, this study shows that TCS11 and TCS12 play a major role in the physiology of *L. casei.* The detailed study of these systems should provide valuable information about their specific roles in order to better understand and improve the performance of this probiotic strain in the different environments it encounters.

REFERENCES

Alcántara, C., A. Revilla-Guarinos*, et al.* (2011). "Influence of Two-Component Signal Transduction Systems of *Lactobacillus casei* BL23 on Tolerance to Stress Conditions." Appl. Environ. Microbiol. **77**(4): 1516-1519.

Azcarate-Peril, M. A., O. McAuliffe*, et al.* (2005). "Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in Lactobacillus acidophilus." Appl. Environ. Microbiol. **71**(10): 5794-5804.

Bäuerl, C., M. Llopis*, et al.* (2013). "*Lactobacillus paracasei* and *Lactobacillus plantarum* strains downregulate proinflammatory genes in an ex vivo system of cultured human colonic mucosa." Genes & Nutrition **8**(2): 165-180.

Bäuerl, C., G. Pérez-Martínez*, et al.* (2010). "Functional analysis of the p40 and p75 proteins from *Lactobacillus casei* BL23." J Mol Microbiol Biotechnol **19**(4): 231-241.

Cui, Y., W. Liu*, et al.* (2012). "A two component system is involved in acid adaptation of *Lactobacillus delbrueckii* subsp. *bulgaricus*." Microbiological Research **167**(5): 253-261.

de Vrese, M. and J. Schrezenmeir (2008). Probiotics, prebiotics, and synbiotics. Adv. Biochem. Eng. Biotechnol. **111:** 1-66.

Hancock, L. E. and M. Perego (2004). "Systematic inactivation and phenotypic characterization of two-component signal transduction systems of *Enterococcus faecalis* V583." J. Bacteriol. **186**(23): 7951-7958.

Hutkins, R. W. a. N., Nancy L. (1993). "pH Homeostasis in Lactic Acid Bacteria." University of Nebraska-Lincoln, Faculty Publications in Food Science and Technology **76**(J Dairy Sci): 2354-2365.

Kashket, E. R. (1987). "Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance." FEMS Microbiology Letters **46**(3): 233-244.

Kawada-Matsuo, M., Y. Yoshida*, et al.* (2011). "Role of two-component systems in the resistance of *Staphylococcus aureus* to antibacterial agents." Virulence **2**(5): 427-430.

Kleerebezem, M. and E. E. Vaughan (2009). "Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity." Annu. Rev. Microbiol. **63**: 269-290.

Landete, J. M., L. García-Haro*, et al.* (2010). "Requirement of the *Lactobacillus casei* MaeKR two-component system for L-malic acid utilization via a malic enzyme pathway." Appl. Environ. Microbiol. **76**(1): 84-95.

Le Breton, Y., G. Boël*, et al.* (2003). "Molecular characterization of *Enterococcus faecalis* two-component signal transduction pathways related to environmental stresses." Environ Microbiol **5**(5): 329-337.

Muller, C., M. Sanguinetti*, et al.* (2008). "Characterization of two signal transduction systems involved in intracellular macrophage survival and environmental stress response in *Enterococcus faecalis*." J Mol Microbiol Biotechnol **14**(1-3): 59-66.

Muñoz-Provencio, D., M. Llopis*, et al.* (2009). "Adhesion properties of *Lactobacillus casei* strains to resected intestinal fragments and components of the extracellular matrix." Arch Microbiol **191**(2): 153-161.

Neuhaus, F. C., M. P. Heaton*, et al.* (1996). "The *dlt* operon in the biosynthesis of D-alanyl-lipoteichoic acid in *Lactobacillus casei*." Microb. Drug Resist. **2**(1): 77-84.

O'Connell-Motherway, M., D. van Sinderen*, et al.* (2000). "Six putative two-component regulatory systems isolated from *Lactococcus lactis* subsp. *cremoris* MG1363." Microbiology **146 (Pt 4)**: 935-947.

Pfeiler, E. A., M. A. Azcarate-Peril*, et al.* (2007). "Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*." J Bacteriol **189**(13): 4624-4634.

Teng, F., L. Wang*, et al.* (2002). "Involvement of PhoP-PhoS homologs in *Enterococcus faecalis* virulence." Infect Immun **70**(4): 1991-1996.

Zilberstein, D., V. Agmon*, et al.* (1982). "The sodium/proton antiporter is part of the pH homeostasis mechanism in *Escherichia coli*." J Biol Chem **257**(7): 3687-3691.

Zilberstein, D., V. Agmon*, et al.* (1984). "*Escherichia coli* intracellular pH, membrane potential, and cell growth." J Bacteriol **158**(1): 246-252.

CHAPTER 2

Ever tried. Ever failed. No matter.
TRY AGAIN.
Fail again. Fail better.

Samuel Beckett

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I —
I took the one less traveled by,
And that has made all the difference.

Robert Frost

CHAPTER 2

Characterization of a Regulatory Network of Peptide Antibiotic Detoxification Modules in *Lactobacillus casei* BL23

Ainhoa Revilla-Guarinos [1], Susanne Gebhard [2], Cristina Alcántara [1], Anna Staroń [2], Thorsten Mascher [2], Manuel Zúñiga [1]

[1] Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC), Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

[2] Ludwig-Maximilians-Universität München, Department Biologie I, Mikrobiologie, Großhaderner Str. 2-4, D-82152, Planegg-Martinsried, Germany

RUNNING TITLE: Peptide Antibiotic Detoxification in *L. casei*

Published in **Applied and Environmental Microbiology**, 2013, 79(10):3160. DOI: 10.1128/AEM.00178-13.

ABSTRACT

Two component systems (TCS) are major signal transduction pathways that allow bacteria to detect and respond to environmental and intracellular changes. A group of TCS has been shown to be involved in the response against antimicrobial peptides (AMPs). These TCS are characterized by the possession of intramembrane sensing histidine kinases, and they are usually associated with ABC transporters of the peptide-7 exporter family (Pep7E). *Lactobacillus casei* BL23 encodes two TCS belonging to this group (TCS09 and TCS12) located next to genes encoding ABC transporters (ABC09 and ABC12) as well as a third Pep7E ABC transporter not genetically associated with any TCS (orphan ABC). This study addressed the involvement of modules TCS09/ABC09 and TCS12/ABC12 in AMP resistance. Results showed that both systems contribute to *L. casei* resistance to AMPs and that each TCS constitutes a functional unit with its corresponding ABC transporter. Analysis of transcriptional levels showed that module 09 is required for the induction of ABC09 expression in response to nisin. In contrast, module 12 controls a wider regulon that encompasses the orphan ABC, the *dlt* operon (D-alanylation of teichoid acids) and the *mprF* gene (L-lysynilation of phospholipids) thus controlling properties of the cell envelope. Furthermore, the characterization of a *dltA* mutant showed that Dlt plays a major role in AMP resistance in *L. casei*. This is the first report on the regulation of the response of *L. casei* to AMPs, giving insight into its ability to adapt to the challenging environments that it encounters as a probiotic microorganism.

INTRODUCTION

Lactobacillus casei is a facultatively heterofermentative lactic acid bacterium commonly found in foodstuffs, plant material and the oral cavity, gastrointestinal tract and genital tract of humans and animals. It is also used as a starter culture in the food industry, and some strains are considered as probiotics (de Vrese and Schrezenmeir, 2008; Kleerebezem and Vaughan, 2009). It is generally agreed that probiotic microorganisms must be able to reach their place of action in a viable and active state in order to exert their beneficial effects on the host. They face a great variety of physicochemical stresses during industrial production, processing and storage, as well as during their passage through the gastrointestinal tract (Corcoran *et al.*, 2008; Kleerebezem and Vaughan, 2009). Furthermore, the intestine is a challenging habitat for probiotics due to competition with the resident microbiota and the action of the host defenses. Among other factors, antimicrobial peptides (AMPs) produced both by the innate immune system of the host (Hancock and Diamond, 2000; Peschel, 2002; Cunliffe, 2003; Dommett *et al.*, 2005; Gallo and Hooper, 2012), and by other microorganisms present in the gastrointestinal tract (Cotter *et al.*, 2005; Gálvez *et al.*, 2007) constitute a major challenge for survival in the intestine.

AMPs usually consist of 12-50 amino acids with a net positive charge of +2 to +7, thus they are called cationic antimicrobial peptides. They are amphipathic molecules which makes them selective towards interaction with and insertion into the negatively charged bacterial membranes (Hancock, 1997). The production of cationic AMPs has been reported in virtually all groups of organisms, including bacteria, fungi, plants and animals (Peschel and Sahl, 2006). Epithelial and immune system cells in the intestine segregate cationic AMPs to the intestinal lumen (Gallo and Hooper, 2012). Together with other immune system effectors, they constitute the first line of innate host defences in the mucosal surfaces (Hancock and Diamond, 2000; Cunliffe, 2003; Dommett *et al.*, 2005; Gallo and Hooper, 2012). In addition to host-produced cationic AMPs, members of the resident microbiota also produce AMPs known as bacteriocins. Bacteriocins are ribosomally synthesized, heat stable antimicrobial peptides produced by bacteria (Cotter *et al.*, 2005; Nishie *et al.*,

2012). They can have a broad or a narrow spectrum of action and the producer strains usually express specific self-protective mechanisms against them (Cotter *et al.*, 2005; Nishie *et al.*, 2012). Most AMPs target essential cell envelope structures (Gallo and Hooper, 2012; Nishie *et al.*, 2012). For example, nisin, a lantibiotic produced by strains of *Lactococcus lactis*, presents a dual mode of antimicrobial activity: it binds with high affinity to the sugar-pyrophosphate moiety of the bacterial cell-wall precursor lipid II and uses it as a docking molecule to cause inhibition of cell wall biosynthesis as well as pore formation in the bacterial membrane (Brötz *et al.*, 1998; Breukink *et al.*, 1999; Wiedemann *et al.*, 2001).

On the other hand, bacteria have evolved mechanisms to detect and elicit a resistance response against AMPs. Among them, two component signal transduction systems (TCS) play a major role in these processes (Guo *et al.*, 1997; Li *et al.*, 2007; Li *et al.*, 2007; Dintner *et al.*, 2011; Staroń *et al.*, 2011). TCS are typically constituted of a membrane-bound histidine kinase that acts as a signal sensor/transducer and a response regulator that usually acts as a transcription activator/repressor (Ninfa and Magasanik, 1986; Nixon *et al.*, 1986; Stock *et al.*, 2000; Gao and Stock, 2009). Histidine kinases monitor environmental signals and in response to a stimulus autophosphorylate at a highly conserved histidine residue (H-box). The high-energy phosphate group is subsequently transferred to an aspartyl residue on the response regulator receiver domain. Phosphorylation of the response regulator in turn modulates the activity of the response regulator effector domain (Gao and Stock, 2009).

A particular group of TCS found in some Firmicutes has been shown to be involved in the response to cell envelope stress exerted mainly by peptide antibiotics (Joseph *et al.*, 2002; Coumes-Florens *et al.*, 2011; Dintner *et al.*, 2011; Gebhard and Mascher, 2011). The histidine kinases of these TCS belong to the intramembrane-sensing histidine kinase subfamily (Mascher, 2006) and are characterized by the possession of two transmembrane helices with a short extracellular linker and no cytoplasmic domains besides the H-box and the kinase domain. These systems are typically associated with ATP-binding cassette (ABC) transporters of the peptide-7 exporter (Pep7E) family (Coumes-Florens *et al.*, 2011; Dintner *et al.*, 2011). These ABC transporters consist of an

ATPase subunit and a permease with 10 transmembrane helices and a large (202 aa) extracellular domain between helices 7 and 8. Interestingly, the characterization of some of the TCS and cognate ABC transporters, such as BceRS/BceAB of *Bacillus subtilis* (Ohki *et al.*, 2003; Bernard *et al.*, 2007; Rietkötter *et al.*, 2008; Staroń *et al.*, 2011) and BceABRS of *Streptococcus mutans* (Ouyang *et al.*, 2010), has shown that the histidine kinase alone is unable to detect the presence of the antimicrobial peptide and that it requires the ABC transporter for signalling. Therefore, TCS and ABC transporters work as fuctional units hereafter termed AMP resistance modules.

A previous survey of the TCS encoded by *L. casei* BL23 identified two gene clusters encoding TCS homologous to BceRS of *B. subtilis*: TCS09 (LCABL_16420/16430) and TCS12 (LCABL_19600/19610). Both of these were found associated with genes encoding putative ABC transporters (ABC09, LCABL_16400/16410 and ABC12, LCABL_19580/19590, respectively) (Fig.1) (Alcántara *et al.*, 2011). In this previous study, inactivation of the corresponding response regulators led to increased sensitivity to antimicrobial peptides such as bacitracin and nisin (Alcántara *et al.*, 2011). Furthermore, it was observed that a strain defective in RR12 displayed a pleiotropic phenotype of greater sensitivity to environmental stresses like presence of bile, acidic pH and high temperatures (Alcántara *et al.*, 2011). Together these data suggested that these systems might be involved in the cell envelope stress response of *L. casei* BL23. This prompted us to investigate in detail the functional role of these TCS and their cognate ABC transporters in *L. casei* and the possible regulatory links between them. The results presented here show that TCS09/ABC09 is a detoxification module involved specifically in resistance against AMPs whereas TCS12/ABC12 controls a larger regulon involved in the maintenance of the physicochemical properties of the cell envelope.

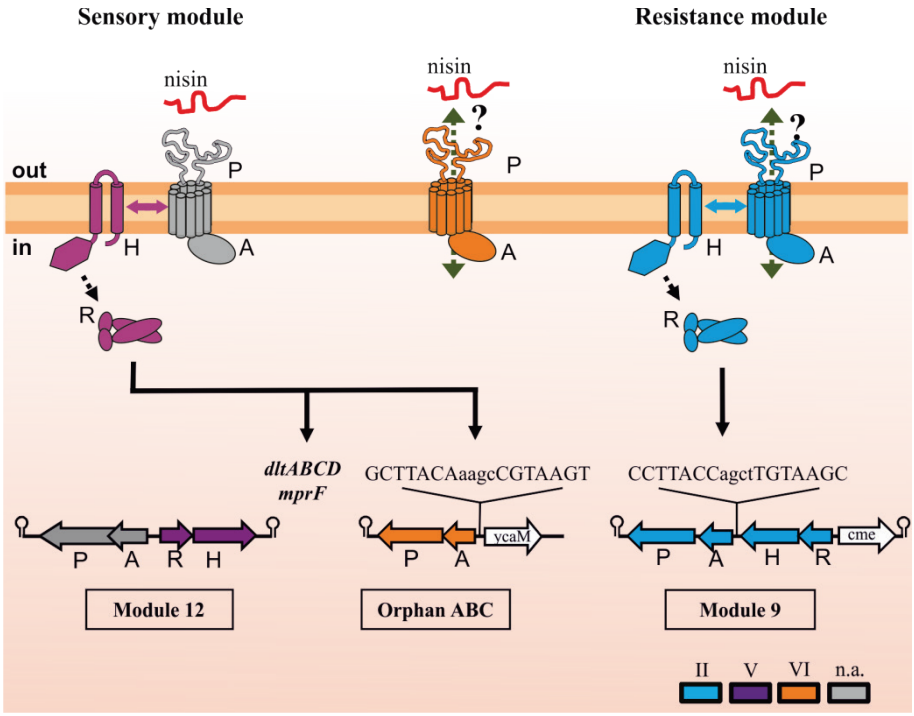


Figure 1. Schematic representation of the signaling network of BceRS/BceAB-like sensory and resistance modules of *L. casei* BL23. TCS and ABC transporters are colored according to their phylogenetic group according to the classification of Dintner *et al.* (Dintner *et al.*, 2011) (n.a., no group assigned). Unrelated genes present in the operons are shown as white arrows. Sequences of putative response regulator binding sites upstream of OrATP and ATP09 are shown; the 7 bp inverted repeats are highlighted in capital letters. Nisin is shown as a substrate interacting with the permeases. Putative transport of nisin by the permeases is shown by green double-head dotted arrows. Signal transfer between TCS09 and ABC09 and between TCS12 and ABC12 is indicated in the membrane bilayer. Putative phosphotransfer between the histidine kinases and response regulators is shown by black dotted arrows. Transcriptional activation is shown by black arrows; additional target genes for module 12 are listed. P, A, R and H stand for permease, ATPase, response regulator and histidine kinase respectively.

MATERIAL AND METHODS

Bacterial strains, plasmids and grown conditions.

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH10B was used as an intermediate host for cloning purposes. *E. coli* strains were grown in LB medium at 37ºC with aeration. *L. casei* strains were grown in MRS broth (Oxoid) at 37ºC under static conditions. The corresponding solid media were prepared by adding 1.5 % (w/v) agar. Strains were stored at -80°C in their corresponding growth media containing 20 % (v/v) glycerol. Antibiotics used were 100 μg ml^{-1} ampicillin for *E. coli* and 5 μg ml^{-1} erythromycin for *L. casei*.

Comparative genomics and motif based search for putative Bce like response regulator binding sites.

The phylogenetic classification of BceRS-like TCS and BceAB-like ABC transporters by Dintner and co-workers (Dintner *et al.*, 2011) included the permeases and histidine kinases of *L. casei* ATCC334 but not those of *L. casei* BL23. Since these two strains are highly homologous (Mazé *et al.*, 2010; Broadbent *et al.*, 2012) we performed a nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/) of the *L. casei* BL23 genome using the previously identified genes of *L. casei* ATCC334 as queries. Genes with 98-99 % identity were identified in all cases (LCABL_16400 to LSEI_1417, LCABL_16420 to LSEI_1419, LCABL_19580 to LSEI_1738, LCABL_19610 to LSEI_1741, LCABL_21670 to LSEI_1993). All *L. casei* BL23 proteins were therefore assigned to the same phylogenetic group as those of *L. casei* ATCC334.

Putative binding sites for the response regulators were identified by a manual search of candidate promoter regions for sequences with similarity to the described binding consensus TNACA-N4-TGTAA (Dintner *et al.*, 2011).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
E. coli DH10B	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*Δ*M15* Δ*lacX74 endA1 recA1 deoR* Δ(*ara,leu*)7697 *araD139 galU galK nupG rpsL* λ⁻	Stratagene
L. casei BL23	Wild-type	B. Chassy, U. Illinois
L. casei ΔRR09	BL23 ΔLCABL_16430	This study
L. casei ΔRR12	BL23 Δ*rrp1* (LCABL_19600)	(Alcántara *et al.*, 2011)
L. casei DLT	LCABL_08550 *(dltA)* mutant; pRV08550 Ery^r	This study
L. casei P09	LCABL_16400 mutant; pRV16400 Ery^r	This study
L. casei P12	LCABL_19580 mutant; pRV19580 Ery^r	This study
L. casei MPRF	LCABL_24490 mutant; pRV24490 Ery^r	This study
pRV300	Insertional vector for *Lactobacillus*, Amp^r, Ery^r	(Leloup *et al.*, 1997)
pRVRR09del	pRV300 containing fused flanking fragments upstream and downstream of LCABL_16430	This study
pRV08550	pRV300 containing a 679-pb internal fragment of LCABL_08550 (*dltA*)	This study
pRV16400	pRV300 containing a 975-pb internal fragment of LCABL_16400	This study
pRV19580	pRV300 containing a 773-pb internal fragment of LCABL_19580	This study
pRV24490	pRV300 containing a 1333-pb internal fragment of LCABL_24490	This study

[a] Amp^r, ampicillin resistance; Ery^r, erythromycin resistance.

Construction of mutants

Standard methods were used for cloning in *E. coli* (Sambrook *et al.*, 1989). Oligonucleotides used in this study are listed in Table S1 in the supplemental material. *E. coli* strains were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad), as recommended by the manufacturer, and *L. casei* strains were transformed as described previously (Posno *et al.*, 1991).

Mutants *L.casei* DLT, MPRF, P09 and P12 were obtained by insertional inactivation of the corresponding genes (see Table 1). Primers were designed to amplify regions of the *L.casei* BL23 genome corresponding to an internal region of each gene. In order to generate knockout mutants in *dltA* and *mprF* genes, the corresponding PCR products were digested with HindIII/SpeI and ligated to the integrative vector pRV300 (Leloup *et al.*, 1997) digested with the same enzymes. For mutants *L.casei* P09 and *L. casei* P12 the PCR products were cloned into pRV300 vector linearized with KpnI using CloneEZ PCR Cloning Kit (GenScript) following the manufacturer´s instructions. The resulting constructs (Table 1) were transformed into *E.coli* DH10B, verified by restriction analysis and used to transform *L. casei* BL23. Single cross-over integrants were selected by resistance to erythromycin, and insertional inactivation of the target gene was confirmed by PCR with a primer hybridizing to an external region to the cloned fragment in the genome and a primer hybridizing to the vector.

In order to obtain a BL23 derived strain harboring a complete deletion of gene LCABL_16430 (RR09), flanking fragments of the region to be deleted were amplified using primer sets RG058/RG059 and RG060/RG061. The resulting PCR products were fused by PCR; the fusion product was digested with EcoRI/ XhoI and ligated into pRV300 digested with the same enzymes. The resulting construct (Table 1) was transformed into *E.coli* DH10B, verified by restriction analysis and by DNA sequencing, and subsequently introduced into *L.casei* BL23 by electroporation. Single cross-over integrations were checked as described before. One single-cross over integrant was grown in MRS without erythromycin for approximately 200 generations in order to obtain a second cross-over recombination leading to the loss of the gene of interest. Cells were

plated on MRS and replica-plated on MRS plus erythromycin. Antibiotic-sensitive clones were isolated, and excision of the pRV300 derivatives leading to deletion of the chromosomal region of interest was checked by PCR and confirmed by sequencing of PCR amplified fragments spanning the deleted regions.

Cytochrome *c* binding assay

Comparison of the whole-cell surface charges of the wild-type strain and mutants ΔRR12, P12, DLT, MPRF, and ΔRR09 was performed by a cytochrome *c* binding assay as described elsewhere (Peschel *et al.*, 1999; Meehl *et al.*, 2007; Matsuo *et al.*, 2011) and modified as follows. Bacterial cells at stationary phase were harvested by centrifugation and washed twice with one volume of 20 mM MOPS (morpholinepropanesulfonic acid) [pH7]. The cells were resuspended in the same buffer at a final concentration of 10^{10} CFU ml^{-1} and incubated with 150 µg ml^{-1} cytochrome c (Sigma) for 10 minutes at room temperature. The mixture was centrifuged twice and the absorbance of the supernatant (containing unbound cytochrome c) was determined at 530 nm. The binding ratio was calculated comparing the absorbance of each supernatant after incubation with the cells, relative to the absorbance of the cytochrome *c* solution without bacterial cells. Results shown are the means and standard deviations from three independent experiments.

Phenotypic characterization of *L. casei* BL23 and mutants

The growth of *L. casei* BL23 and mutants ΔRR12, P12, DLT and MPRF under different stress conditions was determined as previously described (Alcántara *et al.*, 2011) and monitored as optical density at 595 nm (OD_{595}). Briefly, cells from frozen stocks were inoculated onto MRS agar plates and incubated at 37ºC. Single colonies were grown in MRS till stationary phase. Cells were harvested by centrifugation and washed twice with two volumes of 0.1 % (w/v) peptone-water. Cells were inoculated to a final OD_{595} of 0.05 in 250 µl of the corresponding media and dispensed in 96-well microtiter plates. Growth was monitored for 20 h by changes in OD_{595}. The conditions tested were: reference conditions (MRS at 37ºC without shacking), MRS adjusted to pH 4 with HCl, MRS buffered with 0.1 M phosphate buffer (pH 6.8), MRS

supplemented with 0.5% (w/v) bile and growth in MRS at 42ºC. No antibiotics were used in the growth assays. No revertants to the wild-type genotype were detected under these experimental conditions. At least three independent replicates of each growth curve were obtained.

The MIC of the cell wall-acting AMPs bacitracin, mersacidin, nisin, plectasin, vancomycin, and subtilin for all mutants was determined in MRS using different concentrations of the antimicrobial agents. The assays were performed in 96-well microtiter plates inoculated and incubated as indicated above. The MIC was defined as the lowest concentration of antimicrobial agent needed to completely inhibit the growth of the bacterial strain at 15 h. All experiments were performed in duplicate

Reverse-transcription and quantitative real-time PCR (qRT-PCR)

Samples for RNA isolation were collected as follows: overnight cultures from single colonies of *L. casei* BL23 and defective mutants were used to inoculate 100 ml MRS medium to a final OD_{595} of 0.06. Cells were grown at 37ºC to OD_{595} 0.5. Each culture was split into two halves and nisin at a sublethal concentration was added to one half, leaving the other half untreated (control). Incubation was continued for 10 min and subsequently three samples of 10 ml from each culture were taken. The cells were harvested by centrifugation (5000×*g*, 10 min, 4ºC), washed with 1 volume of cold 50 mM EDTA (pH 8.0) and the bacterial pellets were frozen at -80ºC until use. The nisin concentrations used in the induction assays were 22.5 ng ml^{-1} and 750 ng ml^{-1} for *L. casei* BL23, and 22.5 ng ml^{-1} for the defective mutants. Isolation of total RNA from *L. casei* strains, synthesis of cDNA, primer design and qRT-PCR were carried out as described previously (Landete *et al.*, 2010). Primers used are listed in Table S1 in the supplemental material. The *lepA*, *ileS*, *pyrG* and *pcrA* sequences were selected from a set of 10 reference genes (Landete *et al.*, 2010) by using the geNorm application (Vandesompele *et al.*, 2002). The relative expression based on the expression ratio between the target genes and reference genes was calculated using the software tool REST (Pfaffl *et al.*, 2002). Linearity and amplification efficiency were determined for each primer pair. Every real-time PCR determination was performed at least six times.

RESULTS

L. casei BL23 encodes two BceRS-like TCS and three BceAB-like ABC transporters in its genome

Two out of the 17 TCS encoded by *L. casei* BL23 were shown to belong to the Bce-like TCS group: TCS09 and TCS12 (Alcántara *et al.*, 2011). They possess intramembrane-sensing histidine kinases with four (HK09) and nine (HK12) amino acids length extracellular loops between the transmembrane helices. Comparative genomics analyses showed that *L. casei* BL23 possesses three BceAB-like ABC transporters in its genome: ABC09 and ABC12 encoded adjacent to TCS09 and TCS12, respectively, and a third one not genetically associated with any TCS, termed orphan ABC (OrABC) (see Fig.1 and Table S2 in the supplemental material). According to the phylogenetic classification of Dintner *et al.* (Dintner *et al.*, 2011) of BceRS-like TCS and BceAB-like ABC transporters, TCS09 and ABC09 belong to group II, TCS12 belongs to group V, ABC12 could not be assigned to any group and OrABC belongs to group VI. This phylogenetic group assignment raised several questions. On one hand, the fact that both components of module 09 (TCS09 and ABC09) belonged to the same phylogenetic group led us to hypothesize that they could work as a functional unit as has been previously described for homologous modules of *B. subtilis* and *S. aureus* (Gebhard and Mascher, 2011). On the other hand, we wondered if module 12 could also be a functional unit since the signal transfer would occur between a TCS and an ABC from different groups. Finally, OrABC belongs to group VI, which is unique because they are never associated with a TCS. Most of these orphan ABCs contain a putative response regulator binding site in their promoter regions but so far it has not been determined how their expression is regulated. This study aims to provide answers to these questions.

Inactivation of Bce-like TCS systems or their cognate ABC transporters results in increased sensitivity to antimicrobial compounds

The homology of modules 09 and 12 to proteins involved in AMP resistance together with experimental evidence previously obtained strongly

suggested that these systems are likely to be involved in AMP resistance in *L. casei* as well (Alcántara *et al.*, 2011). Furthermore, the genetic organization also suggested that TCS09/ABC09 and TCS12/ABC12 might work as functional units as previously described for other BceAB/BceRS homologous systems. In order to ascertain these points, a collection of mutant strains defective in the response regulators of the TCS and the permease subunits of the cognate ABC transporters were obtained and the sensitivity of the wild-type and all the mutants to AMPs (bacitracin, nisin, mersacidin, plectasin, subtilin and vancomycin) was tested.

Mutant *L. casei* ΔRR12 had been previously obtained (Alcántara *et al.*, 2011). The RR09-encoding gene is located immediately upstream of the HK09 and ABC09 genes (Fig.1). In order to avoid polar effects of the mutation of RR09 on the expression of the genes located downstream, a deletion mutant (*L.casei* ΔRR09) was obtained. Mutants *L. casei* P09 and *L. casei* P12 were constructed by insertional inactivation since the permease 09 and permease 12 encoding genes are located at the end of their corresponding gene clusters (Fig.1), thus polar effects of the insertion on the expression of downstream genes were unlikely.

The sensitivity of the mutants to AMPs was estimated by determining the MIC values of bacitracin, mersacidin, nisin, plectasin, subtilin and vancomycin (Table 2). Strain P09 was more sensitive to bacitracin, nisin, plectasin and subtilin (MIC values were approximately two-fold lower than for the wild-type strain; Table 2). Strain ΔRR09 displayed the same phenotype as P09. These results suggest that TCS09 and ABC09 work together as a functional unit in mediating AMP resistance.

On the other hand, strain P12 was more sensitive than the wild-type strain to all the AMPs tested (MIC values were two fold lower for bacitracin and subtilin, 4-fold lower for vancomycin and mersacidin, 6-fold lower for nisin and 16-fold lower for plectasin; Table 2). Interestingly, mutant ΔRR12 displayed the same phenotype as P12 (Table 2) thus suggesting that they also constitute a functional unit that mediates AMP resistance even though TCS12 and ABC12 do not belong to the same phylogenetic group.

Table 2. MIC values of AMPs against *L. casei* BL23 and derivative strains

	MIC					
Strain	Bacitracin (μg ml^{-1})	Nisin (μg ml^{-1})	Vancomycin (mg ml^{-1})	Plectasin (μg ml^{-1})	Mersacidin (μg ml^{-1})	Subtilin (%)
BL23	10	0.5	1.7	40	10	25
P09	5	0.3	1.7	20	10	10
ΔRR09	5	0.3	1.7	20	10	10
P12	5	0.08	0.4	2.5	2.5	10
ΔRR12	5	0.08	0.4	2.5	2.5	10
DLT	10	0.04	0.4	2.5	2.5	10
MPRF	5	0.5	1.7	>40	5	20

Transcriptional response of *L. casei* BL23 and derived strains to nisin

To further investigate the role of module 09, module 12 and OrABC in AMP resistance, the transcriptional response of the corresponding genes to AMPs was determined by qRT-PCR using nisin as a model AMP. The nisin concentrations used for these assays were chosen so that they had a significant effect on the growth rate but they did not completely inhibit the growth of the strains tested (data not shown). Accordingly, a concentration of 22.5 ng ml^{-1} of nisin was chosen for the mutant strains whereas two concentrations of nisin were used for the wild-type strain, 22.5 ng ml^{-1} and 750 ng ml^{-1}, due to its higher resistance to nisin. Exposure to nisin of exponentially growing cultures of *L. casei* BL23 resulted in a concentration-dependent induction of the expression of ABC09 and OrABC encoding genes, whereas very small changes in the expression of TCS09, TCS12 and ABC12 were detected (Fig.2).

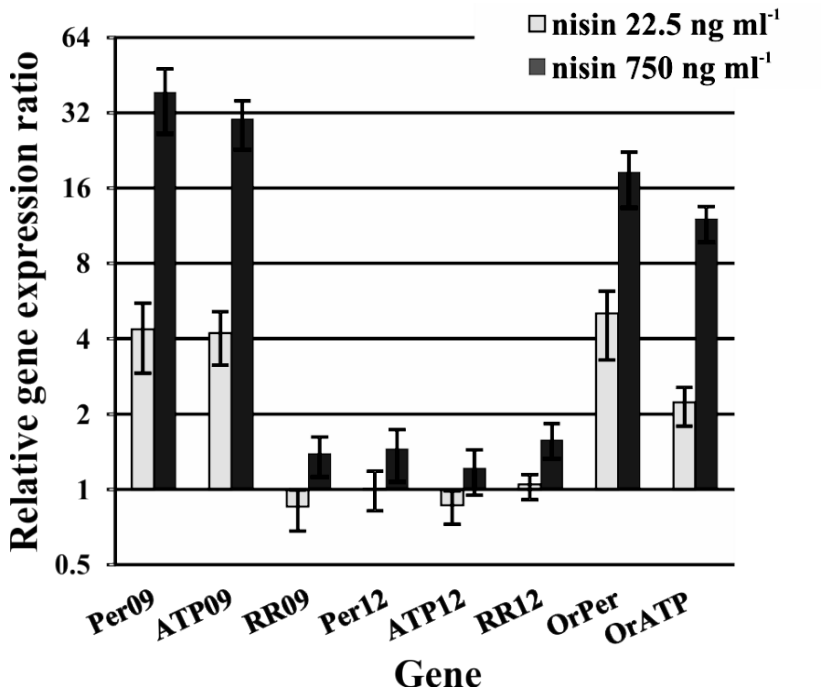


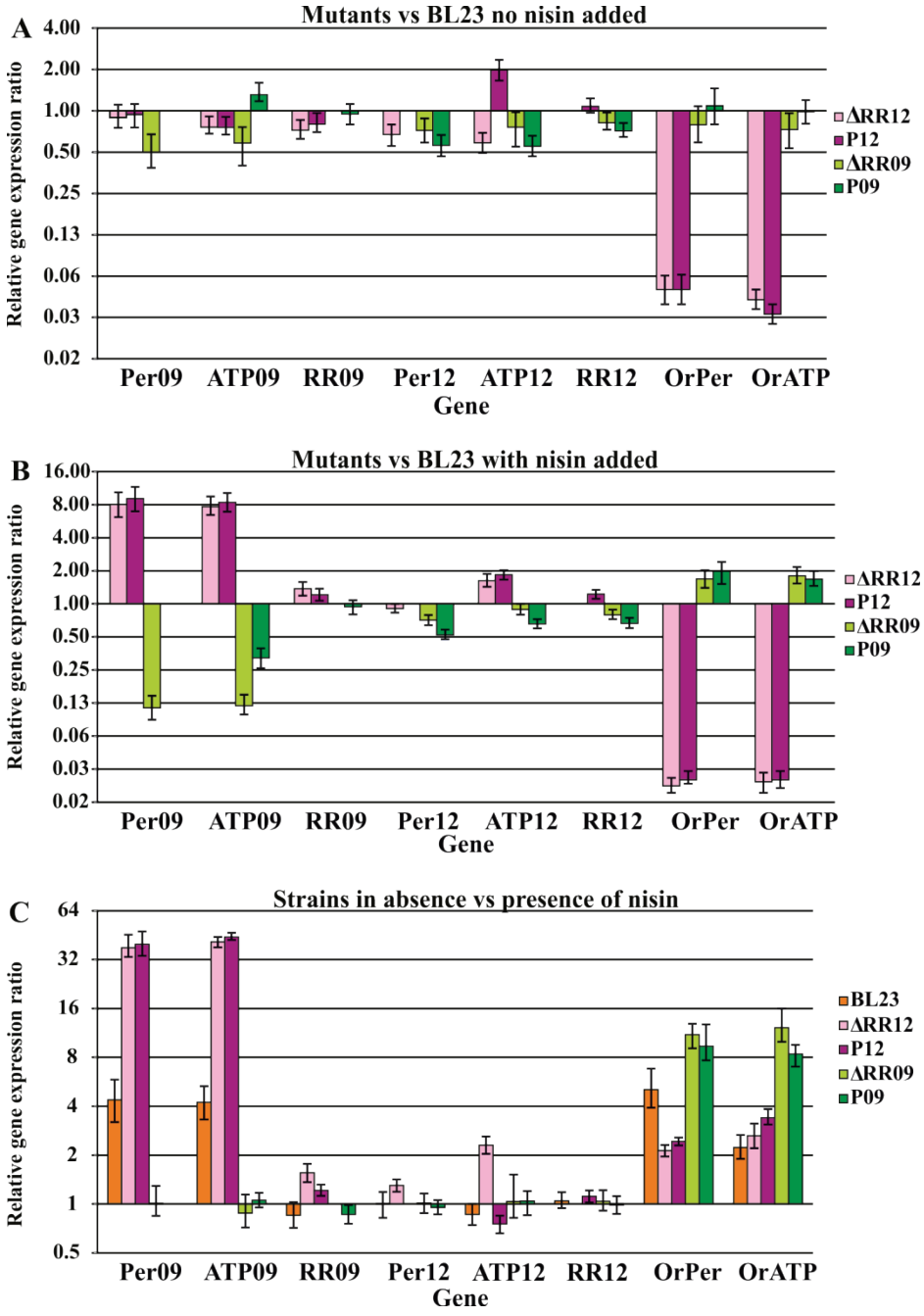
Figure 2. Relative transcript levels of *L.casei* BL23 *bceRS* and *bceAB* homologous genes after nisin induction compared to their expression in the reference condition. Transcript levels were quantified by real-time RT-PCR of RNA samples taken 10 min after nisin addition and compared to samples from untreated cultures. The applied nisin concentrations are indicated. Data are shown as means ± standard deviation from six independent experiments.

Derivative strains ΔRR09 and P09 displayed the same transcriptional profile (Fig.3). In the reference condition, only small differences in transcript levels between both mutant strains and the wild-type strain were observed (Fig. 3A). In contrast, inactivation of either RR09 or permease 09 led to a loss of induction of the ABC09 genes in response to nisin (Fig. 3B and C) whereas the OrABC encoding genes were still induced by nisin. These results indicate that TCS09 is required for the induction of ABC09 observed in the wild-type strain in response to nisin. Furthermore, they also support the hypothesis of ABC09 being part of the signaling pathway possibly acting as the sensory partner of TCS09.

Comparison of strains ΔRR12 and P12 with the wild-type strain showed that both mutations had a similar effect on the gene expression profiles (Fig. 3). In reference conditions (Fig. 3A) a strong decrease in expression of the OrABC encoding genes was observed. This effect was also observed after addition of nisin (Fig. 3B). Furthermore, an eight-fold higher induction of ABC09 encoding genes in response to nisin compared to the parental strain (Fig. 3B and C) was observed in these mutants. The possible reasons for this increased expression are discussed below. In contrast, small differences in the transcript levels of genes encoding RR09, RR12, permease 12 and ATPase 12 were observed compared to the parental strain (Fig. 3 A, B and C). These results show that module 12 is required for the expression of the OrABC and that expression of ABC12 is not under transcriptional control of TCS12.

The identical transcriptional profiles of, on one hand, strains ΔRR09 and P09, and on the other, strains ΔRR12 and P12, are in agreement with the hypothesis that each TCS and ABC couple work together as functional units.

Figure 3. Expression of *bce*-like genes in mutant backgrounds. Relative transcript levels of *bceRS* and *bceAB* homologous genes in *L.casei* BL23 derived strains are shown compared to the parental strain (A) in the absence of nisin and (B) 10 minutes after nisin addition (22.5 ng ml^{-1}). (C) Relative transcript levels of the same genes in *L. casei* BL23 and derivative strains 10 minutes after nisin addition (22.5 ng ml^{-1}), compared to the the same strains in the absence of nisin.


A
Mutants vs BL23 no nisin added
Relative gene expression ratio
4.00
2.00
1.00
0.50
0.25
0.13
0.06
0.03
0.02
Per09
ATP09
RR09
Per12
ATP12
RR12
OrPer
OrATP
Gene
ΔRR12
P12
ΔRR09
P09
B
Mutants vs BL23 with nisin added
Relative gene expression ratio
16.00
8.00
4.00
2.00
1.00
0.50
0.25
0.13
0.06
0.03
0.02
Per09
ATP09
RR09
Per12
ATP12
RR12
OrPer
OrATP
Gene
ΔRR12
P12
ΔRR09
P09
C
Strains in absence vs presence of nisin
Relative gene expression ratio
64
32
16
8
4
2
1
0.5
Per09
ATP09
RR09
Per12
ATP12
RR12
OrPer
OrATP
Gene
BL23
ΔRR12
P12
ΔRR09
P09

Identification of putative target promoters of RR09 and RR12 and verification of the predictions by qRT-PCR

The determination of the transcriptional levels in cells exposed to nisin indicated that module 09 regulated the expression of its cognate transporter ABC09, whereas module 12 regulated the expression of OrABC but not the expression of its cognate ABC12. Furthermore, previous studies had shown that mutant ΔRR12 was sensitive to environmental stresses like presence of bile, acidic pH and high temperature (Alcántara *et al.*, 2011). These findings prompted the question whether TCS12 could also regulate the expression of genes controlling other cellular functions.

In order to identify putative promoters under control of TCS09 or TCS12, a motif based search of the *L. casei* BL23 genome based on the consensus binding sequence for BceR-like response regulators described in Dintner *et al.* (Dintner *et al.*, 2011) was carried out (Fig. 4A). Four putative BceR-like target promoters upstream of genes encoding ATPase09, OrATPase, LCABL_08540 and LCABL_24490 were found in *L. casei* BL23 (see sequence alignment in Fig. 4B). Gene LCABL_08540 is a small gene upstream of the *dlt* operon of *L. casei* BL23 (Mazé *et al.*, 2010), which encodes the molecular machinery for the synthesis of D-alanyl lipoteichoic acids (Neuhaus *et al.*, 1996). LCABL_08540 encodes a putative uncharacterized protein that appears conserved between different *Lactobacillus* strains where it is annotated as a D-Ala-teichoic acid biosynthesis related protein. However, it is not known if it contributes to the functionality of the *dlt* operon in *L. casei* BL23 (Neuhaus *et al.*, 1996) thus we focused our attention on the remaining genes of the *dlt* operon (starting at *dltA*). Gene LCABL_24490 encodes a putative protein significantly similar to characterized lysylphosphatidylglycerol synthetases (Peschel *et al.*, 2001; Thedieck *et al.*, 2006) encoded by *Listeria monocytogenes* (47% identical residues, 68% conserved residues) and *Staphylococcus aureus* (29% identical residues, 52% conserved residues). We have therefore renamed this gene *mprF*.

A

B

```
>P LCABL_16410 (ATPase 9)          GCTACCTTACCAGCTTGTAAGCAACT
>P LCABL_21680 (orphan ATPase)     AGCAGCTTACAAAGCCGTAAGTTACA
>P LCABL_08540 (dlt operon)        CTCATATTACGAAACTGTTAGCTCAT
>P LCABL_24490 (mprF)              ATCACCTTACAAAAAGGTCAGAAAGG
                                     *  **** *    ** **
```

Figure 4. (A) Consensus binding sequence for BceR-like regulators, adapted from Dintner *et al*.(Dintner *et al.*, 2011). (B) Sequence alignment of putative BceR-like target sequences identified in *L. casei* BL23 complete genome by motif based search using consensus sequence shown in (A). See body text for details. Asterisks indicate conserved positions.

The transcriptional levels of *dltA* and *mprF* in *L. casei* BL23 and all derived mutant strains were determined by qRT-PCR in reference conditions and after nisin induction in order to test whether a regulatory link exists between the Bce-like modules and the *dlt* and *mprF* genes of *L. casei* BL23. The expression of *dltA* and *mprF* in reference conditions, relative to the wild-type, was severely decreased in mutants P12 and ΔRR12 (Fig. 5A), whereas no significant differences were found in mutants P09 and ΔRR09. After addition of nisin, expression of *dltA* and *mprF* remained very low compared to the parental strain in strains P12 and ΔRR12 (Fig. 5B). Taken together with the predicted promoter binding sites these data strongly suggest that module 12 regulates the basal expression of *dlt* operon and *mprF* gene in *L. casei* BL23.

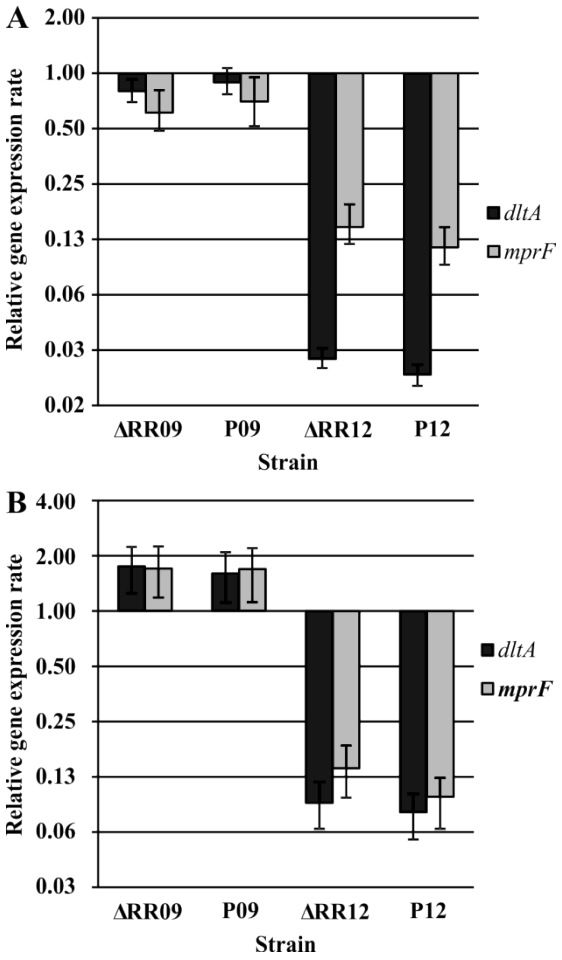


Figure 5 A and B. Expression of *dlt* operon and *mprF* in mutant backgrounds. Relative transcript levels of genes *dltA* and *mprF* in *L.casei* BL23 derivative strains compared to the parental strain (A) in the absence of nisin and (B) 10 minutes after nisin addition (22.5 ng ml^{-1}).

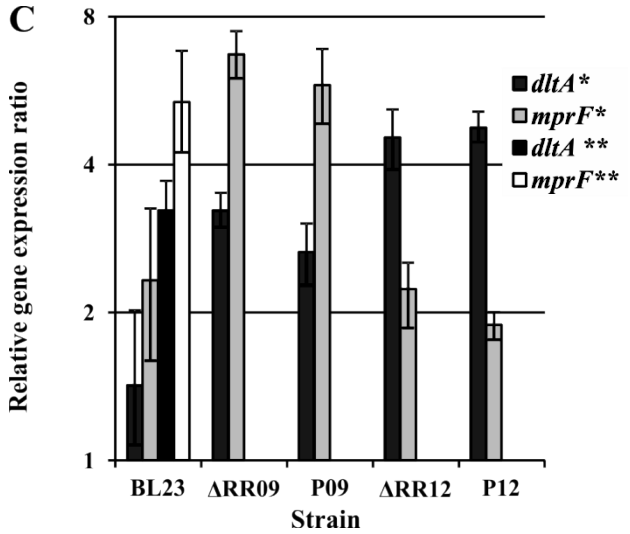


Figure 5 C. Expression of *dlt* operon and *mprF* in mutant backgrounds. (C) Relative transcript levels of *L. casei* BL23 and derivative strains after nisin addition (* 22.5 ng ml^{-1}; ** 750 ng ml^{-1}) compared to the same strains in the absence of nisin.

Interestingly, while the overall expression levels were much lower in mutants P12 and RR12 compared to the wild-type, nisin-dependent induction of both the *dlt* operon (4.5- fold approx.) and *mprF* gene (2-fold approx.) relative to reference conditions was still observed (Fig. 5C). These data suggest that *L. casei* must harbor an additional regulatory system to control their expression.

The pleiotropic phenotype of module 12 defective strains is due to a low expression of the *dlt* operon

We then addressed the question whether the pleiotropic phenotype previously described for ΔRR12 mutant (Alcántara *et al.*, 2011) was due to reduced expression of MrpF or Dlt. To this end two new mutant strains were obtained by insertional inactivation of *mprF* and *dltA*: *L. casei* MPRF and *L. casei* DLT, respectively (Table 1). Growth of these two strains under different conditions was compared to the wild-type strain and mutants ΔRR12 and P12

as previously described (Alcántara *et al.*, 2011). Mutant MPRF showed the same phenotype as the wild-type in all conditions tested (Fig. 6). However, mutants DLT, P12 and ΔRR12 did not reach the same final OD_{595} as the wild-type in reference conditions (Fig. 6A), were more sensitive to 0.5 % bile and high temperature (Fig. 6 B and C) than the wild-type, and they were not able to grow at pH 4 (Fig. 6D). To ascertain if the lower final OD under reference conditions was due to a higher sensitivity to acid, the bacteria were grown in MRS supplemented with 0.1 M phosphate buffer, thus preventing acidification of the medium during growth. Under these conditions, mutants DLT, P12 and ΔRR12 were able to reach similar final OD_{595} values as the wild-type (Fig. 6E). This result demonstrates that the different growth in reference conditions is mainly due to their higher acid sensitivity.

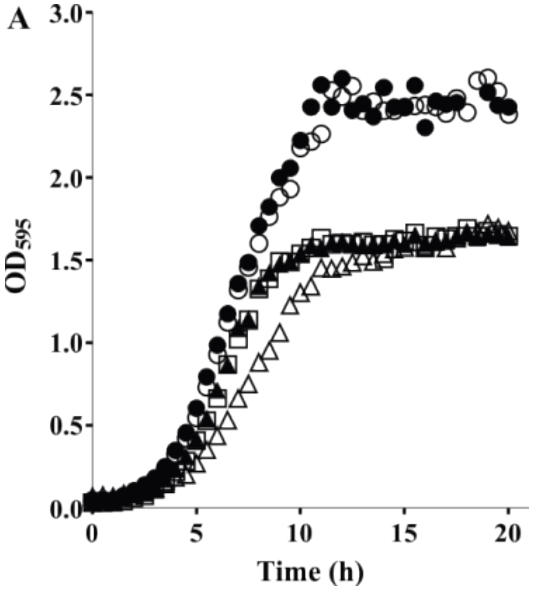


Figure 6A Growth of *L. casei* BL23 (●) and strains ΔRR12 (□), Per12 (▲), DLT (Δ) and MPRF (○) under reference conditions. Data are shown as representative results of three independent experiments.

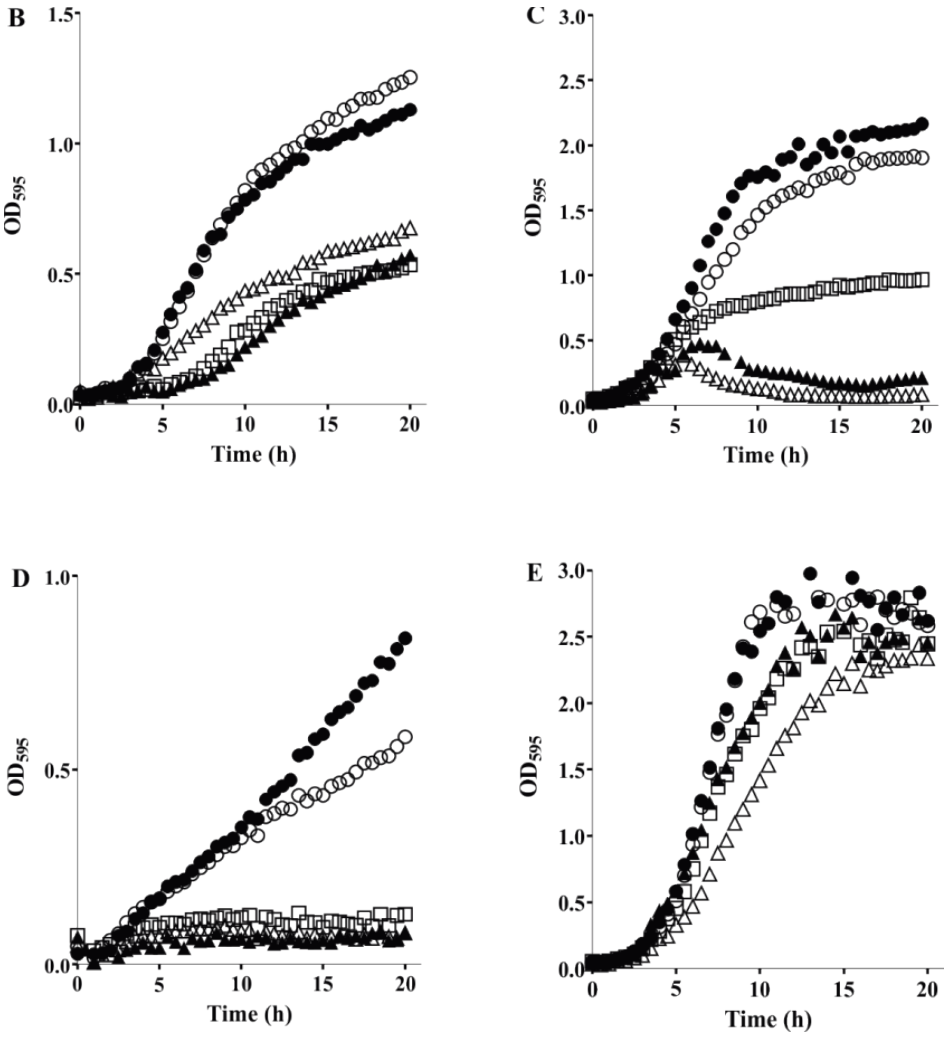


Figure 6 B, C, D and E. Growth of *L. casei* BL23 (●) and strains ΔRR12 (□), Per12 (▲), DLT (Δ) and MPRF (○) under different conditions. (B) In the presence of 0.5 % bile. (C) 42 ºC. (D) Medium adjusted to pH 4. (E) Buffered media (MRS supplemented with 0.1 M phosphate). Data are shown as representative results of three independent experiments.

The MIC values of the previously assayed AMPs against the strains DLT and MPRF were also determined (Table 2). The MIC values of nisin, vancomycin, plectasin, mersacidin and subtilin obtained for the DLT mutant were similar to the MIC values for mutants P12 and ΔRR12 (Table 2). In contrast, strain MPRF showed similar MICs as the wild-type with only a slight increase in sensitivity to bacitracin and mersacidin (Table 2).

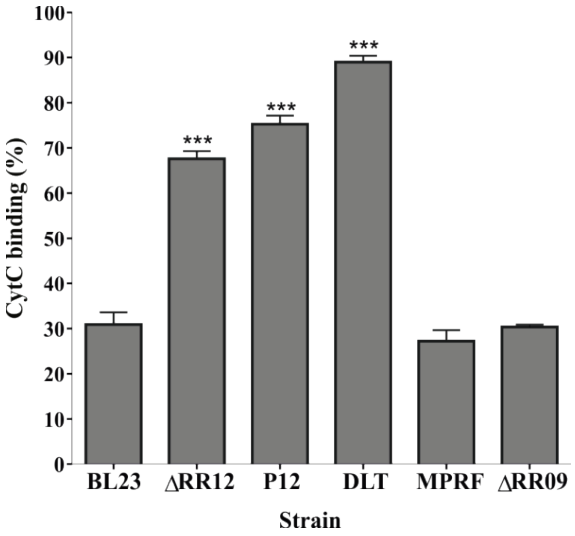


Figure 7. Cytochrome c binding assay. Binding is expressed as a percentage of bound cytochrome c to stationary phase cells of strain BL23 and derivatives after incubation with 150 µg ml^{-1} cytochrome c for 10 minutes at room temperature. Data represent the means and standard deviations from three independent experiments. Asterisks indicate means significantly different ($P<0.05$, unpaired Student *t* test) of the indicated strain compared to the control strain (BL23).

Since Dlt and MprF activities modulate the bacterial cell surface charge and thus affect the susceptibility to AMPs (Peschel *et al.*, 1999; Matsuo *et al.*, 2011), a cytochrome c (Cyt *c*) binding affinity assay was performed in order to determine whether strains DLT and MPRF actually displayed a difference in cell surface charge compared to the wild-type strain. Cyt *c* is a highly positively charged protein and its binding to the bacterial surface depends on the net

negative charge. *L. casei* BL23 and strain ΔRR09 bound ~30% of the Cyt *c* present in the solution (Fig. 7). Strains ΔRR12, P12 and DLT bound 67.5%, 75% and 89% respectively, of the Cyt *c* present in the solution (Fig. 7), indicating that they have a more negative surface than the wild-type strain. However, the *mprF* mutant showed the same phenotype than the wild-type strain (Fig. 7).

Considering together these results, we conclude that the pleiotropic phenotype of module 12 mutants is due to the low expression of the *dlt* operon that leads to an increase in the net negative charge of the bacterial surface.

DISCUSSION

AMPs are key components of the innate immune defense system (Jenssen *et al.*, 2006), but bacteria also produce AMPs in order to gain an advantage in complex microbial communities such as the gut microbiome (Dobson *et al.*, 2012). Conversely, bacteria have evolved sophisticated systems to counteract the action of AMPs. BceRS/BceAB modules have been shown to be involved in AMP resistance in different Firmicutes bacteria (Li *et al.*, 2007; Rietkötter *et al.*, 2008; Dintner *et al.*, 2011; Hiron *et al.*, 2011; Kawada-Matsuo *et al.*, 2011; Staroń *et al.*, 2011; Falord *et al.*, 2012). In this study we showed that the BceRS/BceAB-like modules of *L. casei* BL23, TCS09/ABC09 and TCS12/ABC12, are also involved in AMP resistance in this probiotic microorganism. Inactivation of either RR09 or permease 09 led to higher sensitivity to bacitracin, nisin, plectasin and subtilin than that observed in the wild-type strain, whereas inactivation of either RR12 or permease 12 resulted in higher sensitivity to bacitracin, nisin, mersacidin, plectasin, subtilin and vancomycin (Table 2). In this study we have used nisin as a model AMP. Nisin belongs to the group of lantibiotics, ribosomally synthesized peptides which are characterized by lanthionine or methyllanthionine rings among other post-translational modifications (Schnell *et al.*, 1988; Sahl and Bierbaum, 1998; Lubelski *et al.*, 2008). Mature nisin is an elongated, amphipatic and positively charged molecule and it is active against Gram positive bacteria including lactobacilli. Nisin-producing strains possess immunity conferred to them by the lipoprotein NisI and the ABC transporter NisFEG (Siegers and Entian, 1995). NisFEG does not belong to the peptide-7 exporter family so that it is not related to BceAB-like ABC transporters. Strong synergy between NisI and NisFEG has been observed indicating that both proteins are required for immunity (Lubelski *et al.*, 2008).

An important feature of Bce-like modules characterized to date is that the transporters are required for stimulus perception and signal transduction within the modules (Bernard *et al.*, 2007; Coumes-Florens *et al.*, 2011; Dintner *et al.*, 2011; Hiron *et al.*, 2011). The results reported in this study showed that the mutant strains defective in the response regulator and its associated ABC permease from the same module displayed identical phenotypes and changes

in transcript levels (Table 2 and Figs. 3 and 5). These results strongly suggest that both TCS09 and TCS12 require their respective ABC transporters and that therefore these TCS and ABC transporters work as functional modules as it has been previously described for other systems of this group (Bernard *et al.*, 2007; Rietkötter *et al.*, 2008; Ouyang *et al.*, 2010; Hiron *et al.*, 2011).

Determination of changes in transcript levels in response to nisin showed that the genes coding for ABC09 but not RR09, are induced in *L.casei* BL23 in response to nisin in a concentration dependent manner (Fig. 2). This induction was lost in the mutant strains ΔRR09 and P09 (Fig 3 B and C). These results show that TCS09 regulates the nisin-dependent expression of ABC09 and strongly suggest that both the TCS and the ABC transporter are required for nisin induction of ABC09 expression. Our findings also suggest that ABC09 confers resistance to the compound that induces its expression, similarly to what has been described for the ABC transporters BceAB and PsdAB of *B. subtilis* (Staroń *et al.*, 2011). Therefore, taking into consideration the evidence provided by other homologous systems and the results obtained in this study, we propose that the target AMP (nisin) is sensed by ABC09 and activates HK09 which in turn transfers the signal to RR09 resulting in the induction of the expression of the ABC09 that confers the resistance (Fig. 1).

On the other hand, small changes in response to nisin were detected in the transcript levels of the genes coding for ABC transporter 12 as well as the RR12 gene (Fig. 2). Since a transporter solely involved in substrate sensing but not in transport does not necessarily need to increase its expression in response to the presence of the substrate (Dintner *et al.*, 2011), this result suggests that module 12 acts as a sensory system without detoxification function. Furthermore, the transcript levels of the genes encoding the orphan ABC transporter, the *dlt* operon and the *mprF* gene in Per12 mutant were lowered to the same basal levels as in the ΔRR12 mutant, both in reference conditions (Fig. 3A and 5A) and in response to nisin (Fig 3B and 5B), relative to the wild type. These results further support that ABC12 is as essential as TCS12 for controlling the constitutive expression of the *dlt* operon, gene *mprF* and the orphan ABC encoding genes but apparently not for a specific response to nisin. This idea is further substantiated by the fact that expression of genes

controlled by module 12 is still induced in module 12 defective mutants (Figs. 3C and 5C) although their transcript levels are much lower than in the parental strain. These data suggest that other regulatory systems besides module 12 control the expression of the *dlt* operon and *mprF* in *L. casei* BL23.

The involvement of BceRS/BceAB modules in the control of the cell envelope charge has been previously described in other organisms. For example, the GraRS TCS of *S. aureus* and the homologous system ApsRS of *Staphylococcus epidermidis* control the expression of the *dlt* operon and *mprF*, as well as *vraFG* which encodes a BceAB-like transporter that also mediates the AMP resistance (Herbert *et al.*, 2007; Li *et al.*, 2007; Li *et al.*, 2007; Falord *et al.*, 2012). The TCS VirRS of *Listeria monocytogenes* also controls expression of the *dlt* operon and *mprF* among other genes (Mandin *et al.*, 2005). Interestingly, VirRS apparently does not regulate the expression of its adjacent ABC transporter Lmo1747-1746 (Mandin *et al.*, 2005) but it regulates the expression of the non-genetically associated ABC transporter AnrAB which has also been shown to be involved in AMP resistance (Collins *et al.*, 2010).

Initial binding of cationic AMPs to bacterial cell envelopes is mediated by electrostatic attraction between the positively charged peptides and the negatively charged bacterial surfaces (Peschel and Sahl, 2006). Bacteria can utilize a number of mechanisms to modulate their cell envelope charge. Many Gram-positive bacteria can neutralize polyanionic teichoic acid polymers of the cell wall by esterification with D-alanine (Neuhaus and Baddiley, 2003). The machinery required for this activity is encoded by the *dlt* operon. The MprF protein is found in both Gram-positive and Gram-negative bacteria. It catalyzes the modification of the anionic phospholipids of the membrane with L-lysine or L-alanine (Ernst and Peschel, 2011). MprF and the DltABCD system thus function as bacterial immune evasion systems that confer resistance to cationic AMPs by reducing the negative net charge of bacterial cell surface (Peschel *et al.*, 1999; Neuhaus and Baddiley, 2003; Thedieck *et al.*, 2006; Li *et al.*, 2007; Li *et al.*, 2007; Ernst *et al.*, 2009; Ernst and Peschel, 2011; Matsuo *et al.*, 2011). Besides the sensitivity to antimicrobial peptides, other phenotypes have been associated with a non-functional Dlt system. For example, inactivation of *dltC* in *Streptococcus mutans* resulted in the loss of the acid

tolerance response and lower growth rate than the parental strain (Boyd *et al.*, 2000).

The Cyt *c* binding assay showed that low expression of the *dlt* operon in *L. casei* effectively led to an increase in the net negative charge of the bacterial surface in mutants ΔRR12, P12 and DLT (Fig. 7). These strains also displayed lower AMP resistance and growth defects (Table 2 and Fig. 6). On the contrary, low expression of MprF did not significantly change the cell surface charge (Fig. 7) and only resulted in a two-fold decrease in MIC for bacitracin and mersacidin (Table 2), whereas its growth was similar to the wild-type strain under all other conditions tested (Fig.6). Ernst and colleagues (Ernst *et al.*, 2009) reported that the level of lysyl-phosphatidylglycerol (Lys-PG) did not correlate with the level of cationic AMP resistance since a basal amount of Lys-PG is enough to confer full cationic AMP resistance. Together these results indicate that the growth defects observed in module 12 defective mutants were mainly due to low expression of the *dlt* operon. In this sense, it is worth noting the higher expression of ABC09 in response to nisin in the module 12 defective mutants compared to the wild-type strain (Fig. 3 B). A possible explanation for this observation is that the higher cell surface negative charge of these mutants would increase their affinity for nisin and thus increase the signal for induction of ABC09 although additional evidence is needed to ascertain this hypothesis.

In conclusion, results reported in this study show that the BceRS/BceAB-like modules of *L. casei* BL23, TCS09/ABC09 and TCS12/ABC12, are involved in AMP resistance in this probiotic microorganism (Fig.1). It is also shown that BceRS-like TCS of *L. casei* BL23 constitute functional modules with their cognate ABC transporters. Module 09 regulates the expression of its cognate ABC09 that possibly has a dual function of sensing and resistance to AMPs. Module 12 is an AMP sensory system where ABC12 does not have a direct role in AMP detoxification. The sensitivity of module 12 mutants to AMPs and their pleiotropic phenotype is caused by reduced expression of the RR12 regulated genes, in particular the *dlt* operon. The function of OrABC, the third target operon of module 12, remains unclear. To our knowledge this is the first report

on the characterization of a complete set of AMP resistance mechanisms in the probiotic species *L. casei*.

ACKNOWLEDGMENTS

This work was financed by funds from the former Spanish Ministry of Science and Innovation (AGL2007-60975, AGL2010-15679 and Consolider Fun-C-Food CSD2007-00063) and Generalitat Valenciana (ACOMP2012/137). A. Revilla-Guarinos was the recipient of an FPI grant (BES-2008-004527) from the former Spanish Ministry of Science and Innovation. Work in the lab of T. Mascher was financed by a grant from the Deutsche Forschungsgesellschaft (MA2837/1-3). S. Gebhard is funded by grants from the Deutsche Forschungsgesellschaft (GE2164/3-1) and the Fonds der Chemischen Industrie.

We thank Amalia Blasco and Tina Wecke for technical assistance.

SUPPLEMENTAL MATERIAL

Table S1. Primers used in this study.

Primer	Gene	Sequence (5´- 3´) [a]
Cloning		
RG037	LCABL_08550	AGTCAAGCTTGTTCAGATTATTCGCGCACC
RG038	LCABL_08550	GACTACTAGTCTGACACTTGATTGCCTTGC
RG039	LCABL_24490	AGTCAAGCTTTTCCGCTATCGTCTGTCTTG
RG040	LCABL_24490	GACTACTAGTTGAAGGTCGTCTAGCAAAGC
RG047	LCABL_16400	**CTATAGGGCGAATTG**GGTACCGCAAGCCTTCAGTATCGCCG
RG048	LCABL_16400	**CTCGAGGGGGGGCCC**GGTACCTCAGCCGCGTTTTGATAGCG
RG049	LCABL_19580	**CTATAGGGCGAATTG**GGTACCGCATTGCTGGCGTCGTTTTG
RG050	LCABL_19580	**CTCGAGGGGGGGCCC**GGTACCGCCGGTCGGGAAAAGATTGG
RG058	LCABL_16440	TTTTCTCGAGTAAAATATAACCAGCCAACAGCGG
RG059	LCABL_16440/ LCABL_16430 intergenic region and LCABL_16420	CGAGCGGCAATAAGCTTTCATCATCTCGTATACATCCCCTTGGGTATC
RG060	LCABL_16420 and intergenic region LCABL_16440 / LCABL_16430	GATACCCAAGGGGATGTATACGAGATGATGAAAGCTTATTGCCGCTCG
RG061	LCABL_16420	AAAAGAATTCTGTCACTAATAACAGTTGGCAATGG
Cloning checking		
pRV300.fw	pRV300 plasmid	GTTTTCCCAGTCACGAC
pRV300.rv	pRV300 plasmid	CAGGAAACAGCTATGAC
RG003	LCABL_19580	GTCCGATCACTGACAAGC
RG066	LCABL_16420	GCAATTGTTCAAAAATAAAATGCAGCC
RG067	LCABL_16440	GATGGCCAACGCGGCAGATAAG
RG074	LCABL_16440	CATATTGCAACCCGGCGAGA
RG075	LCABL_16420	CACCGCGTTTGGGGAAAATG
LSEI1418R	LCABL_16410	GTCAACATTACTTAAATTAAAAA

Table S1. Primers used in this study (*continuation*).

Primer	Gene	Sequence (5´- 3´) [a]
qRT-PCR		
lepA-F	lepA	CACATTGATCACGGGAAGTC
lepA-R	lepA	GTAATGCCACGTTCACGTTC
ileS-F	ileS	ACCATTCCGGCTAACTATGG
ileS-R	ileS	TCAGGATCTTCGGATTTTCC
pcrA-F	pcrA	CGGCCAATAATGTGATTCAG
pcrA-R	pcrA	TCATCAGTTTCGCTTTGAGC
pyrG-F	pyrG	AATTGCGCTTTTCACTGATG
pyrG-R	pyrG	CGAAATGATCGACCACAATC
RG006	LCABL_19580	GGGAACGCGCATTCATTGTG
RG007	LCABL_19580	TCTCGCGCTGAACAAGATCC
RG008	LCABL_21670	TTGCCGGTATTTTGGTCGGG
RG009	LCABL_21670	ATGTCCACAATACGGCTGGC
RG019	LCABL_08550	TGGTCGAGGTTTTCTTGGGC
RG020	LCABL_08550	CCGGTGTATGGGCAACATCC
RG021	LCABL_24490	GCCGGATCAGCCAAGACTTG
RG022	LCABL_24490	TTAGCATCGGTGTAACGGCG
RG027	LCABL_19590	TAGCTTTCAGGTCAACGCGG
RG028	LCABL_19590	CTTGGCGTCTCAATCGTTGC
RG029	LCABL_19600	GGCAATGAATATGGGCGCTG
RG030	LCABL_19600	TAGGTTCGTCGAAGCAAGGC
RG031	LCABL_21680	CACCCGCATTGAAAGGTGTC
RG032	LCABL_21680	GCAAGGTCGTTTTCCCTGAAC
RG033	LCABL_16410	GGACAGGATCTGAGCAACGTC
RG034	LCABL_16410	ATTGAAGGTGTCAAGCAAGTCG
RG054	LCABL_16400	GTACCGTCCTTTCCCGCATC
RG055	LCABL_16400	CCGATGGTAATGATCCCGGC
RG056	LCABL_16430	AGCGAGTTACGCAAACACAG
RG057	LCABL_16430	CGGCTCCTAAGTTCATCGCC

[a] Restriction sites are underlined. Sequences highlighted in boldface type are the sequences for the CloneEZ PCR reaction. See materials and methods for details.

Table S2. BceRS-BceAB like resistance modules identified in *L. casei* BL23

	Descriptive name in this article	Loci	Phylogenetic group[a]	Induced by nisin?	Resistance substrates[b]	ABC regulated by TCS?	ABC required for sensing?
Resistance module	TCS09: *hk09-rr09*	LCABL_16420-16430	II	No	Bac, Nis, Plec, Sub	Yes (TCS09)	Yes
	ABC09: *permease09 –ATPase09*	LCABL_16400-16410	II	Yes			
	TCS12*: rr12-hk12*	LCABL_19600-19610	V	No	Bac, Nis, Mer, Plec, Sub, Van	No	Yes
	ABC12: *permease12-ATPase12*	LCABL_19580-19590	No group assigned	No			
	Orphan ABC*: orphan permease-orphan ATPase*	LCABL_21670-21680	VI	Yes	n.d.	Yes (TCS12)	n.d.

[a]Phylogenetic group assigned according to Dintner *et al. 2011* (Dintner *et al.*, 2011) classification of Pep7E-type permeases and BceS-like HKs.

[b] Bac, bacitracin; Nis, nisin; Mer, mersacidin; Plec, plectasin; Sub, subtilin; Van, vancomycin.

n.d. not determined

REFERENCES.

Alcántara, C., A. Revilla-Guarinos*, et al.* (2011). "Influence of two-component signal transduction systems of *Lactobacillus casei* BL23 on tolerance to stress conditions." Appl. Environ. Microbiol. **77**(4): 1516-1519.

Bernard, R., A. Guiseppi*, et al.* (2007). "Resistance to bacitracin in *Bacillus subtilis*: unexpected requirement of the BceAB ABC transporter in the control of expression of its own structural genes." J. Bacteriol. **189**(23): 8636-8642.

Boyd, D. A., D. G. Cvitkovitch*, et al.* (2000). "Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity." J. Bacteriol. **182**(21): 6055-6065.

Breukink, E., I. Wiedemann*, et al.* (1999). "Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic." Science **286**(5448): 2361-2364.

Broadbent, J. R., E. C. Neeno-Eckwall*, et al.* (2012). "Analysis of the *Lactobacillus casei* supragenome and its influence in species evolution and lifestyle adaptation." BMC Genomics **13**: 533.

Brötz, H., M. Josten*, et al.* (1998). "Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics." Mol. Microbiol. **30**(2): 317-327.

Collins, B., N. Curtis*, et al.* (2010). "The ABC transporter AnrAB contributes to the innate resistance of *Listeria monocytogenes* to nisin, bacitracin, and various beta-lactam antibiotics." Antimicrob. Agents Chemother. **54**(10): 4416-4423.

Corcoran, B. M., C. Stanton*, et al.* (2008). "Life under stress: the probiotic stress response and how it may be manipulated." Curr. Pharm. Des. **14**(14): 1382-1399.

Cotter, P. D., C. Hill*, et al.* (2005). "Bacteriocins: developing innate immunity for food." Nat. Rev. Microbiol. **3**(10): 777-788.

Coumes-Florens, S., C. Brochier-Armanet*, et al.* (2011). "A new highly conserved antibiotic sensing/resistance pathway in Firmicutes involves an ABC transporter interplaying with a signal transduction system." PLoS One **6**(1): e15951.

Cunliffe, R. N. (2003). "Alpha-defensins in the gastrointestinal tract." Mol. Immunol. **40**(7): 463-467.

de Vrese, M. and J. Schrezenmeir (2008). "Probiotics, prebiotics, and synbiotics." Adv. Biochem. Eng. Biotechnol. **111**: 1-66.

Dintner, S., A. Staroń*, et al.* (2011). "Coevolution of ABC transporters and two-component regulatory systems as resistance modules against antimicrobial peptides in *Firmicutes* Bacteria." J. Bacteriol. **193**(15): 3851-3862.

Dobson, A., P. D. Cotter*, et al.* (2012). "Bacteriocin production: a probiotic trait?" Appl. Environ. Microbiol. **78**(1): 1-6.

Dommett, R., M. Zilbauer*, et al.* (2005). "Innate immune defence in the human gastrointestinal tract." Mol. Immunol. **42**(8): 903-912.

Ernst, C. M. and A. Peschel (2011). "Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids." Mol. Microbiol. **80**(2): 290-299.

Ernst, C. M., P. Staubitz*, et al.* (2009). "The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion." PLoS Pathog. **5**(11): e1000660.

Falord, M., G. Karimova*, et al.* (2012). "GraXSR proteins interact with the VraFG ABC transporter to form a five-component system required for cationic antimicrobial peptide sensing and resistance in *Staphylococcus aureus*." Antimicrob. Agents Chemother. **56**(2): 1047-1058.

Gálvez, A., H. Abriouel*, et al.* (2007). "Bacteriocin-based strategies for food biopreservation." Int. J. Food Microbiol. **120**(1-2): 51-70.

Gallo, R. L. and L. V. Hooper (2012). "Epithelial antimicrobial defence of the skin and intestine." Nat. Rev. Immunol. **12**(7): 503-516.

Gao, R. and A. M. Stock (2009). "Biological insights from structures of two-component proteins." Annu. Rev. Microbiol. **63**: 133-154.

Gebhard, S. and T. Mascher (2011). "Antimicrobial peptide sensing and detoxification modules: unravelling the regulatory circuitry of *Staphylococcus aureus*." Mol. Microbiol. **81**(3): 581-587.

Guo, L., K. B. Lim*, et al.* (1997). "Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*." Science **276**(5310): 250-253.

Hancock, R. E. and G. Diamond (2000). "The role of cationic antimicrobial peptides in innate host defences." Trends Microbiol. **8**(9): 402-410.

Hancock, R. E. W. (1997). "Peptide antibiotics." The Lancet **349**(9049): 418-422.

Herbert, S., A. Bera*, et al.* (2007). "Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci." PLoS Pathog. **3**(7): e102.

Hiron, A., M. Falord*, et al.* (2011). "Bacitracin and nisin resistance in *Staphylococcus aureus*: a novel pathway involving the BraS/BraR two-component system (SA2417/SA2418) and both the BraD/BraE and VraD/VraE ABC transporters." Mol. Microbiol. **81**(3): 602-622.

Jenssen, H., P. Hamill*, et al.* (2006). "Peptide antimicrobial agents." Clin. Microbiol. Rev. **19**(3): 491-511.

Joseph, P., G. Fichant*, et al.* (2002). "Regulatory relationship of two-component and ABC transport systems and clustering of their genes in the *Bacillus/Clostridium* group, suggest a functional link between them." J. Mol. Microbiol. Biotechnol. **4**(5): 503-513.

Kawada-Matsuo, M., Y. Yoshida*, et al.* (2011). "Role of two-component systems in the resistance of *Staphylococcus aureus* to antibacterial agents." Virulence **2**(5): 427-430.

Kleerebezem, M. and E. E. Vaughan (2009). "Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity." Annu. Rev. Microbiol. **63**: 269-290.

Landete, J. M., L. García-Haro*, et al.* (2010). "Requirement of the *Lactobacillus casei* MaeKR two-component system for L-malic acid utilization via a malic enzyme pathway." Appl. Environ. Microbiol. **76**(1): 84-95.

Leloup, L., S. D. Ehrlich*, et al.* (1997). "Single-crossover integration in the *Lactobacillus sake* chromosome and insertional inactivation of the *ptsI* and *lacL* genes." Appl. Environ. Microbiol. **63**(6): 2117-2123.

Li, M., D. J. Cha*, et al.* (2007). "The antimicrobial peptide-sensing system aps of *Staphylococcus aureus*." Mol. Microbiol. **66**(5): 1136-1147.

Li, M., Y. Lai*, et al.* (2007). "Gram-positive three-component antimicrobial peptide-sensing system." Proc. Natl. Acad. Sci. U. S. A. **104**(22): 9469-9474.

Lubelski, J., R. Rink*, et al.* (2008). "Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin." Cell. Mol. Life Sci. **65**: 455-476.

Mandin, P., H. Fsihi*, et al.* (2005). "VirR, a response regulator critical for *Listeria monocytogenes* virulence." Mol. Microbiol. **57**(5): 1367-1380.

Mascher, T. (2006). "Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in Firmicutes bacteria." FEMS Microbiol. Lett. **264**(2): 133-144.

Matsuo, M., Y. Oogai*, et al.* (2011). "Growth-phase dependence of susceptibility to antimicrobial peptides in *Staphylococcus aureus*." Microbiology **157**(Pt 6): 1786-1797.

Mazé, A., G. Böel*, et al.* (2010). "Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23." J. Bacteriol. **192**(10): 2647-2648.

Meehl, M., S. Herbert*, et al.* (2007). "Interaction of the GraRS two-component system with the VraFG ABC transporter to support vancomycin-intermediate resistance in *Staphylococcus aureus*." Antimicrob. Agents Chemother. **51**(8): 2679-2689.

Neuhaus, F. C. and J. Baddiley (2003). "A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria." Microbiol. Mol. Biol. Rev. **67**(4): 686-723.

Neuhaus, F. C., M. P. Heaton*, et al.* (1996). "The *dlt* operon in the biosynthesis of D-alanyl-lipoteichoic acid in *Lactobacillus casei*." Microb Drug Resist **2**(1): 77-84.

Ninfa, A. J. and B. Magasanik (1986). "Covalent modification of the *glnG* product, NRI, by the *glnL* product, NRII, regulates the transcription of the *glnALG* operon in *Escherichia coli*." Proc. Natl. Acad. Sci. U. S. A. **83**(16): 5909-5913.

Nishie, M., J. Nagao*, et al.* (2012). "Antibacterial peptides "bacteriocins": an overview of their diverse characteristics and applications." Biocontrol Sci. **17**(1): 1-16.

Nixon, B. T., C. W. Ronson*, et al.* (1986). "Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes *ntrB* and *ntrC*." Proc. Natl. Acad. Sci. U. S. A. **83**(20): 7850-7854.

Ohki, R., Giyanto*, et al.* (2003). "The BceRS two-component regulatory system induces expression of the bacitracin transporter, BceAB, in *Bacillus subtilis*." Mol. Microbiol. **49**(4): 1135-1144.

Ouyang, J., X. L. Tian*, et al.* (2010). "The BceABRS four-component system regulates the bacitracin-induced cell envelope stress response in *Streptococcus mutans*." Antimicrob. Agents Chemother. **54**(9): 3895-3906.

Peschel, A. (2002). "How do bacteria resist human antimicrobial peptides?" Trends Microbiol. **10**(4): 179-186.

Peschel, A., R. W. Jack*, et al.* (2001). "*Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine." J. Exp. Med. **193**(9): 1067-1076.

Peschel, A., M. Otto*, et al.* (1999). "Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides." J. Biol. Chem. **274**(13): 8405-8410.

Peschel, A. and H. G. Sahl (2006). "The co-evolution of host cationic antimicrobial peptides and microbial resistance." Nat. Rev. Microbiol. **4**(7): 529-536.

Pfaffl, M. W., G. W. Horgan*, et al.* (2002). "Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR." Nucleic Acids Res. **30**(9): e36.

Posno, M., R. J. Leer*, et al.* (1991). "Incompatibility of *Lactobacillus* Vectors with Replicons Derived from Small Cryptic *Lactobacillus* Plasmids and Segregational Instability of the Introduced Vectors." Appl. Environ. Microbiol. **57**(6): 1822-1828.

Rietkötter, E., D. Hoyer*, et al.* (2008). "Bacitracin sensing in *Bacillus subtilis*." Mol. Microbiol. **68**(3): 768-785.

Sahl, H. G. and G. Bierbaum (1998). "Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria." Annu. Rev. Microbiol. **52**: 41-79.

Sambrook, J., E. F. Fritsch*, et al.* (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Laboratory Press.

Schnell, N., K. D. Entian*, et al.* (1988). "Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings." Nature **333**(6170): 276-278.

Siegers, K. and K. D. Entian (1995). "Genes involved in immunity to the lantibiotic nisin produced by *Lactococcus lactis* 6F3." Appl. Environ. Microbiol. **61**(3): 1082-1089.

Staroń, A., D. E. Finkeisen*, et al.* (2011). "Peptide antibiotic sensing and detoxification modules of *Bacillus subtilis*." Antimicrob. Agents Chemother. **55**(2): 515-525.

Stock, A. M., V. L. Robinson*, et al.* (2000). "Two-component signal transduction." Annu. Rev. Biochem. **69**: 183-215.

Thedieck, K., T. Hain*, et al.* (2006). "The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*." Mol. Microbiol. **62**(5): 1325-1339.

Vandesompele, J., K. De Preter*, et al.* (2002). "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes." Genome Biol. **3**(7): RESEARCH0034.

Wiedemann, I., E. Breukink*, et al.* (2001). "Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity." J. Biol. Chem. **276**(3): 1772-1779.

CHAPTER 3

Mais s´il n´y avait pas de difficultés, où serait l´amusement.

Gustave Flaubert

If I find 10.000 ways something won´t work,
I haven´t failed. I am not discouraged,
because every wrong attempt discarded is another step forward.

Thomas Edison

CHAPTER 3

Characterization of the response to low pH of *Lactobacillus casei* ΔRR12, a mutant strain with low D-alanylation activity and sensitivity to low pH

Ainhoa Revilla-Guarinos [1], Cristina Alcántara [1], Nicolas Rozès [2], Birgit Voigt [3], Manuel Zúñiga [1]

[1] Dpto. Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC) Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain
[2] Dpt. Bioquímica i Biotecnología, Facultat d'Enologia, Universitat Rovira i Virgili, Campus Sescelades, c/ Marcel·lí Domingo, s/n, Tarragona 43007, Spain
[3] Institute for Microbiology, University of Greifswald, D-17487 Greifswald, Germany

RUNNING TITLE: Response to low pH of *L. casei* ΔRR12

Published in **Journal of Applied Microbiology**, 2014. DOI:10.1111/jam.12442.

ABSTRACT

Aims: To identify the differences that account for the acid sensitivity of *Lactobacillus casei* ΔRR12. RR12 controls the expression of the *dlt* operon and its inactivation leads to a diminished teichoic acid D-alanylation activity. To this end, a comparison of the response of ΔRR12 to low pH with the parental strain *L. casei* BL23 was carried out.

Methods and Results: The ability to induce an acid tolerance response, fatty acid composition and proteome changes induced in both strains in response to acid were investigated. Results obtained showed that both strains induce a growth phase dependent acid tolerance response. However, significant differences in the content of fatty acids and membrane associated proteins were detected.

Conclusions: The greater abundance of cytoplasmic proteins in the membrane fraction of the mutant strain ΔRR12 suggests an increased permeability of the cell membrane in this strain.

Significance and Impact of the Study:

The analysis of the response to low pH of strain ΔRR12 indicated that the inactivation of TCS12 affected the content of fatty acids and proteins associated to the cell envelope. Increased abundance of cytoplasmic proteins suggested that low alanylation of teichoic acids affected the permeability of the cell membrane and possibly accounts for the acid sensitivity of strain ΔRR12.

Keywords: *Lactobacillus casei*, acid sensitivity, acid tolerance response, fatty acids, D-alanylation of teichoics acids

INTRODUCTION

Lactic acid bacteria (LAB) are Gram positive, non-sporulating fermentative bacteria that produce lactic acid as the major end-product of carbohydrate metabolism so that proliferation of LAB is generally accompanied by acidification of the surrounding medium. Bacteria maintain a pH gradient (ΔpH) across the membrane so that the cytoplasmic pH (pH_{in}) remains more alkaline than the medium pH (pH_{out}) (Booth, 1985; Kashket, 1987). As the medium pH decreases, the ratio of undissociated forms of organic acids increases. Undissociated acids can enter the cell resulting in the acidification of the cytoplasm. If the cell cannot compensate this proton influx, ΔpH cannot be maintained and cellular functions are impaired (Booth, 1985; Kashket, 1987; Hutkins and Nannen, 1993). In order to survive in an acidic environment, several mechanisms contribute to regulate the homeostasis of pH_{in} in LAB and the response to acid stress . These mechanisms include proton pumps, mainly the F_0F_1-ATPase, decarboxylases acting on amino acids such as glutamic acid or organic acids (for example, malic acid), deiminases acting on amino acids or amines such as arginine or putrescine, and urease (for reviews see (Hutkins and Nannen, 1993; van de Guchte *et al.*, 2002; Cotter and Hill, 2003; De Angelis and Gobbetti, 2004; Corcoran *et al.*, 2008)). Response to acid stress in LAB has also been associated to upregulation of chaperones and other stress responsive proteins (Hamilton and Svensäter, 1998; Lim *et al.*, 2000; De Angelis *et al.*, 2001; Frees *et al.*, 2003). However, the analysis of the transcriptional response of *Lactobacillus casei* to acid stress showed that acid adaptation in this organism involved the triggering of the stringent response with concomitant downregulation of components of the translational apparatus as well as a number of chaperones and other stress-related proteins (Broadbent *et al.*, 2010). Involvement of stringent response in acid resistance had also been suggested for *Lactococcus lactis* (Rallu *et al.*, 2000). In addition, a number of studies have shown that LAB change the fatty acid composition of their membranes in response to acidification of the medium (Fozo *et al.*, 2004; Fozo and Quivey, 2004; Broadbent *et al.*, 2010). Modulation of the surface cell properties via D-alanylation of teichoic acids also plays a major role in acid resistance in LAB as evidenced by the increased acid sensitivity observed in Dlt-

defective mutants (Boyd *et al.*, 2000; Vélez *et al.*, 2007; Revilla-Guarinos *et al.*, 2013).

A number of regulatory mechanisms involved in acid resistance have been characterized in LAB (van de Guchte *et al.*, 2002; Cotter and Hill, 2003; Corcoran *et al.*, 2008). Among them, signal transduction two component systems (TCS) have been shown to play a major role. For example, inactivation of two response regulators of *Lactobacillus sakei* led to mutants that were more acid-sensitive than the parental strain (Morel-Deville *et al.*, 1998). Inactivation of a TCS possibly involved in the regulation of the proteolytic apparatus of *Lactobacillus acidophilus* also resulted in an acid-sensitive phenotype (Azcarate-Peril *et al.*, 2005). A survey of the phenotypic effect of TCS inactivation in *L. casei* identified six TCS whose inactivation led to growth defects at acidic pH (Alcántara *et al.*, 2011). Growth defects in acid media associated to TCS inactivation have also been described in *Lc. lactis* (O'Connell-Motherway *et al.*, 2000), *Streptococcus pyogenes* (Ichikawa *et al.*, 2011) and *Streptococcus suis* (Han *et al.*, 2012) among others. These results indicate that TCS modulate the acid resistance ability of many LAB although the mechanisms by which they exert their effect remain to be determined in many cases.

Lactobacillus casei is a facultative heterofermentative LAB naturally found in food (dairy and meat products, fermented vegetables, etc.) and the oral cavity, gastrointestinal and genital tracts of humans and other animals. *L. casei* is an organism of industrial interest for its use as a starter culture for some dairy products and, because some strains are considered as probiotics (de Vrese and Schrezenmeir, 2008; Kleerebezem and Vaughan, 2009). Probiotic microorganisms are currently the focus of an intense research effort that aims to determine their possible health benefits. A number of these studies have concluded that probiotics must survive the transit through the gastrointestinal tract. Survival in this environment depends, among other factors, on its ability to survive to acid (Hutkins and Nannen, 1993; Walter and Ley, 2011). We previously reported that strain ΔRR12, a *Lactobacillus casei* BL23 mutant lacking the response regulator of TCS12, displayed a premature arrest of growth and it was very sensitive to low pH (Alcántara *et al.*, 2011). A subsequent study showed that this defect was mainly due to low expression of

the *dlt* operon and it could be alleviated by increasing the buffering capacity of the growth medium (Revilla-Guarinos *et al.*, 2013). These results suggested that TCS12 could be involved in the mechanisms that allow *L. casei* BL23 to adapt to acidic environments. To further investigate this hypothesis, the acid tolerance and acid stress responses of *L. casei* BL23 and ΔRR12 were compared.

MATERIAL AND METHODS

Bacterial strains and growth conditions

L. casei BL23 and the derivative strain ΔRR12 (Alcántara *et al.*, 2011) were routinely grown in deMan Rogosa Sharpe (MRS) broth (Oxoid) at 37 °C without shaking. Agar was added at 1.8% for plates. Cells were stored at -80 °C in MRS medium supplemented with 15% (v/v) glycerol.

Acid tolerance response (ATR) assays were carried out as follows: cells from the stock cultures were inoculated on MRS agar plates. For the growth curves at 37 °C and ATR induction assays, single colonies of each strain were used to inoculate three 5 ml aliquots of MRS medium and the cultures were incubated overnight at 37 °C. Each culture was used to inoculate 50 ml batches of pre-warmed MRS medium at optical density of 0.05 at 595 nm (OD_{595}). Incubation was continued at 37 °C and two samples of 1 ml were withdrawn at selected time points. Both sample cells were washed with one volume of 0.05% peptone-water. Subsequently they were resuspended in either one volume of peptone-water (control) or one volume of MRS adjusted to pH 2.5 with HCl (acid challenge). Incubation of acid-challenged cells was continued at 37 °C for 45 minutes. Survival after acid challenge was determined by comparing viable cell countings in the control samples and acid-challenged samples. Significant differences were estimated by using Student's t-test (2-tail unpaired with significance measured at a probability level of $P<0.05$) as implemented in Graphpad Prism version 5.

Determination of cell membrane fatty acid composition

Three independent 5 ml overnight cultures of *L. casei* BL23 and derivative strain ΔRR12 were used to inoculate 6 batches of 200 ml of MRS broth at OD_{595} 0.1. Cultures were incubated at 37 °C and changes in OD_{595} and pH were monitored. When the cultures media reached pH values of 4.7, 4.3 and 4, two samples of each culture corresponding to 20 OD units were taken, washed twice with water and kept frozen at -80 °C until use. The extraction of fatty acids (FA) was carried out as previously described (Rozès *et al.*, 1993).

Briefly, methanolysis of cell materials was carried out with 500 µl of 1 mol l^{-1} sodium methoxide in methanol. Heptanoic acid methyl ester (C7:0) and heptadecanoic acid methyl ester (C17:0) were added to the samples as internal standards. The preparations were mixed for 1 min and then FA methyl esters were extracted by shaking with 100 µl hexane for 30 seconds. Samples were pelleted and the upper phase was recovered for analaysis on a Hewlett-Packard 6850 gas chromatograph (Agilent Technologies). Two microliters were injected (splitless, 0.75 min) into an HP-FFAP column (Agilent Technologies) with an HP 6850 automatic injector. The initial temperature was set at 100 ºC and increased by 3.5 ºC/min up to 240 ºC during 10 min. The injector and detector temperatures were set at 220 and 260 ºC, respectively. The carrier gas was helium at a flow rate of 1 ml/min. Relative amounts of FAs were calculated from their respective chromatographic peak areas. These values were related to the dry weight of cells and expressed as a percentage of the total FA extracted.

Two-dimensional SDS-PAGE analysis of the response of *L. casei* BL23 and ΔRR12 to acid challenge

Single colonies of *L. casei* BL23 and ΔRR12 were used to inoculate three 5 ml aliquots of MRS medium and the cultures were incubated overnight at 37 °C. Each culture was used to inoculate two 250 ml batches of pre-warmed MRS medium at OD_{595} 0.06 for the treatment or control assays. Incubation was continued to OD_{595} 0.6. At this point, 250 ml of pre-warmed MRS was added to the control samples and 250 ml of MRS adjusted to pH 3.6 with HCl was added to the treated cultures until a final pH of 4.4 was reached. Incubation was continued for 45 min at 37 °C. At this point, the cultures were collected and the proteins associated to membrane fractions were purified as previously described (Alcántara and Zúñiga, 2012). Two biological replicates and three technical replicates of each biological replicate were analyzed for each growth condition. 2D gel electrophoresis and analysis were performed as described previously (Rivas-Sendra *et al.*, 2011). A protein was considered to be under- or overproduced when, after image analysis and subsequent computing of the normalized spot volumes, the means from at least four gels were 1·5-fold

different among the conditions tested at a significance level of $P < 0{\cdot}05$ (Student's *t*-test for paired samples).

Selected spots were excised from 2D gels and transferred to polypropylene tubes containing ultrapure water. Proteins were identified by MALDI-TOF/TOF after trypsinolysis at the Institute of Microbiology (University of Greifswald) according to Wolff et al. (Wolf *et al.*, 2008). MALDI-MS and MS/MS data were searched using the Mascot search engine (version 2.1.0.4) with the Uniprot database (http://www.uniprot.org/uniprot/?query=Lactobacillus+casei+BL+23&sort=score&format=*).

RESULTS

The mutant strain ΔRR12 can induce a growth phase-dependent acid tolerance response (ATR)

One of the mechanisms that protect bacteria from acid killing is the adaptive ATR: a brief exposure of the cells to a sublethal extracellular pH induces physiological changes that allow the bacteria to survive to the subsequent exposure to a lethal extracellular pH (Goodson and Rowbury, 1989). In order to determine whether the higher sensitivity of ΔRR12 strain to acid was due to a defective ATR response, survival after an acid challenge of *L. casei* BL23 and ΔRR12 was determined. Previous studies of the laboratory showed that an acid challenge at pH 2.5 for 45 minutes was lethal for *L. casei* BL23 at early stages of growth; however the cells were able to survive to this acid challenge in the final stages of growth (data not shown). Thus pH 2.5 was chosen for the ATR induction studies. Cells from cultures in MRS at 37 °C of *L. casei* BL23 and ΔRR12 were withdrawn as the growth medium reached predetermined pH values. They were transferred to MRS medium adjusted to pH 2.5 with HCl and incubated at 37 °C for 45 minutes. Survival after the acid challenge was determined by viable counting in MRS agar plates. Results obtained showed that both strains elicit a growth phase-dependent ATR as evidenced by their increased survival after the acid challenge (Table 1). Induction of ATR occurred at the end of the exponential growth phase in the wild-type strain (Fig. 1). However, induction of ATR was delayed in strain ΔRR12 compared to the wild-type strain BL23 (Fig. 1 and Table 1). In this experiment, a similar trend was observed in the evolution of the pH for the mutant strain and BL23 and the same final pH was reached by both strains at the end of the assay. This observation indicated that ΔRR12 impaired growth was not due to loss of metabolic activity. Taken together, these observations suggested that inactivation of TCS12 did not impair ATR induction but it could be interfering cellular processes involved in the adaptation to grow at low pH of *L. casei*.

Table 1. Growth-phase dependent ATR induction in *L. casei* BL23 and ΔRR12*. ATR is represented by the percentage of survival of the acid challenged cells (incubation for 45 minutes at 37ºC in MRS adjusted to pH 2.5 with HCl) relative to the control non-acid challenged cells. OD_{595} and pH of the cultures at the time of acid challenge is indicated.

BL23			ΔRR12		
OD_{595}	**pH**	**% survival**	**OD_{595}**	**pH**	**% survival**
0.82±0.02	5.31±0.03	$1.52\times10^{-5}\pm2.6\times10^{-5}$	0.91±0.03	5.08±0.04	$7.4\times10^{-6}\pm4\times10^{-6}$
2.51±0.14	4.76±0.02	$4.52\times10^{-4}\pm5.3\times10^{-4}$	2.42±0.05	4.66±0.03	$5.1\times10^{-5}\pm0.8\times10^{-5}$
3.72±0.04	4.44±0.02	2.17±0.85	3.23±0.07	4.36±0.02	$4.4\times10^{-3}\pm2.4\times10^{-3}$
6.77±0.03	3.71±0.01	65.29±1.00	4.61±0.21	3.90±0.01	72.80±4.8

* Means and standard deviations of three independent determinations.

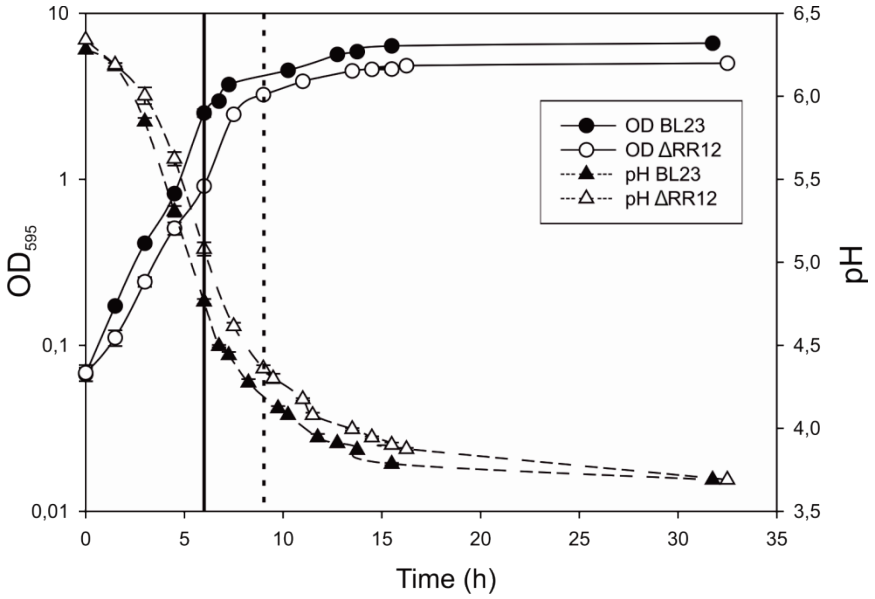


Figure 1. Variation of OD_{595} and medium pH during growth of *L. casei* BL23 and its derivative strain ΔRR12 in MRS at 37 ° C. The vertical solid and dotted lines indicate the sampling points at which ATR induction was detected for strains BL23 and ΔRR12, respectively. Values represent the means of three independent experiments; error bars represent standard deviations.

Variation of the cell membrane fatty acid composition

In order to determine whether the FA composition of the membrane of *L. casei* BL23 changes during growth and if this change is dependent on TCS12, the FA composition of BL23 and ΔRR12 strains was analyzed. For this purpose, cells were grown in MRS at 37 °C and samples were withdrawn for FA determination when the pH of the culture media reached 4.7, 4.3 and 4. Results obtained are shown in Table 2. In both strains, the major FAs were palmitic (C16 30-38% of total FA approximately), oleic (C18:1 c9; 13-24%), vaccenic (C18:1 c11; 15-16%) and dihydrosterculic (C19 cyc9; 9-17%) acids. Significant differences between strains were observed at the three pH assayed for palmitic, oleic, dihydrosterculic and lactobacillic (C19 cyc11) acids (Table 2). No significant differences were observed at any pH value only for cis-11-hexadecenoic acid (C16 c11; Table 2).

Table 2. Variation of fatty acid composition of *L. casei* BL23 and ΔRR12 during growth*. Fatty acids: myristic (C14), myristoleic (C14 c9), palmitic (C16), palmitoleic (C16 c9), cis-11-hexadecenoic (C16 c11), stearic (C18), oleic (C18 c9), vaccenic (C18 c11), and the cyclopropanes dihydrosterculic (C19 cyc9) and lactobacillic acid (C19 cyc11).

	pH 4.7			pH 4.3			pH 4.0		
FA	BL23	ΔRR12	P value†	BL23	ΔRR12	P value	BL23	ΔRR12	P value
C14	6.70±0.14	6.37±0.67	0.262	6.38±0.42	5.80±0.62	0.089	7.34±1.02	5.78±0.43	**0.006**
C14 c9	0.82±0.03	0.74±0.07	**0.045**	0.74±0.05	0.75±0.25	0.433	0.83±0.06	0.58±0.10	**4.57×10^{-4}**
C16	31.01±0.39	34.27±1.14	**5.86×10^{-5}**	32.70±2.10	37.31±0.92	**6.05×10^{-4}**	30.18±0.89	37.60±2.79	**9.96×10^{-5}**
C16 c9	6.97±0.09	7.34±0.17	**0.001**	7.37±0.10	7.42±0.22	0.594	7.87±0.28	6.98±0.31	**4.24×10^{-4}**
C16 c11	0.73±0.02	0.78±0.02	0.574	0.84±0.05	0.86±0.08	0.681	0.87±0.06	0.81±0.02	0.055
C18	0.95±0.02	1.13±0.14	**0.022**	0.91±0.06	1.04±0.03	**0.001**	0.99±0.19	1.02±0.08	0.679
C18 c9	24.46±0.48	21.30±0.67	**3.98×10^{-5}**	18.25±1.47	15.01±0.97	**0.001**	16.05±1.21	13.07±2.88	**0.041**
C18 c11	15.11±0.26	16.49±0.99	**0.013**	16.37±0.43	16.22±1.36	0.796	15.57±1.03	14.74±0.82	0.155
C19 cyc9	11.00±0.41	9.03±0.79	**1.37×10^{-4}**	13.28±0.76	11.86±0.30	**0.002**	16.67±1.71	14.29±0.58	**0.009**
C19 cyc11	1.46±0.12	1.68±0.12	**0.013**	2.62±0.36	3.45±0.32	**0.002**	3.22±0.31	4.54±1.03	**0.013**

* Results are expressed as the mean percentage of each FA species over the total FA pool ± standard deviation. Six independent samples of each strain were used.

† T test. Differences statistically significant ($p<0.05$) are highlighted in bold.

The FA content varied with external pH in both strains. As the medium pH decreased, the content of unsaturated FAs decreased whereas the content of cyclic FAs increased in both strains (Fig. 2). However, whereas the content of unsaturated FAs always was higher than that of saturated FAs in the wild-type strain, as pH decreased, the content of saturated FAs exceeded that of unsaturated FAs in the mutant strain ΔRR12 (Fig. 2).

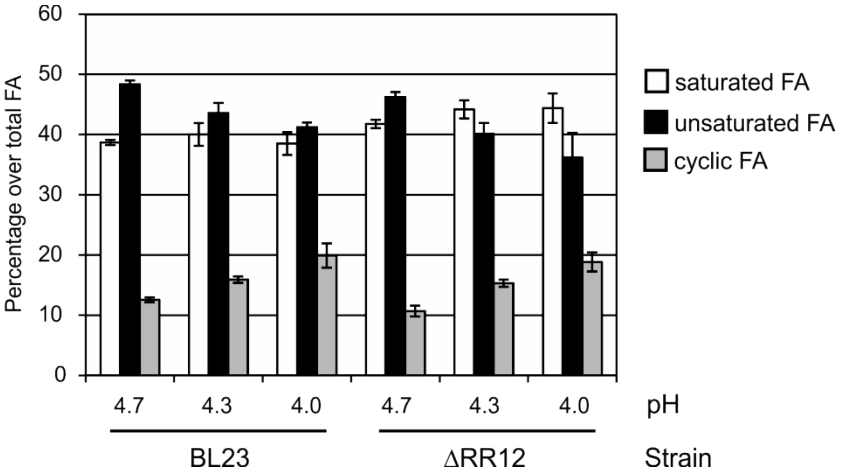


Figure 2. Variation of the percentage of fatty acid classes of strains BL23 and ΔRR12 during growth. Values represent the means of six independent determinations; error bars represent standard deviations.

Considering the individual FAs, a significant increase in the ratios of dihydrosterculic acid and lactobacillic acid was observed in both strains ($p<0.05$) whereas the oleic acid ratio significantly decreased ($p<0.05$; Table 2). The evolution of other FA varied depending on the strain considered. For example, a significant increase in the palmitic acid content and a decrease in the myristic and myristoleic acids content as the pH decreased were observed in strain ΔRR12 whereas no significant changes were observed in the parental strain (Table 2). In contrast, the content of palmitoleic acid increased in strain BL23 whereas no significant changes were observed in strain ΔRR12. These results show that the FA content of strain ΔRR12 differs significantly of that of

the parental strain BL23 and the changes in the content of some FA in response to decreasing medium pH also differed between strains.

Proteomic analysis of the response of *L. casei* BL23 and ΔRR12 to sublethal acidic pH exposition

To gain insight into the possible role of TCS12 in the control of the ATR of *L. casei*, a proteomic analysis was carried out. Fig. 3 shows representative gels resulting from the analysis of the membrane-associated proteins. When the samples subjected to acid pH challenge were compared with the reference samples, 15 and 17 spots were significantly more abundant in *L. casei* BL23 and ΔRR12, respectively whereas one and two spots were less abundant in *L. casei* BL23 and ΔRR12, respectively. Furthermore, comparison of ΔRR12 with the wild-type strain BL23 showed that, 6 proteins were more abundant and two less abundant in the mutant strain relative to the wild-type strain in reference conditions. In response to acid, 9 proteins were more abundant and 4 less abundant in the mutant relative to the wild-type strain. 32 spots could be identified by MS; four of them (spots LC07, LC08, LC30, LC31) had a double identification. Another four spots did not render reliable mass spectra. Calculated fold changes are listed in Table 3 and the quantitative data of the spots identified are shown in Table S1 in the Supplemental Material at the end of the chapter.

Identified proteins were putatively involved in a wide variety of cellular functions, thus indicating that acid challenge induced global changes in *L. casei* BL23 physiology. The changes in the abundance of some proteins in both strains suggest that these proteins are involved in the response to acid of *L. casei* BL23. On the other hand, the abundance of some proteins varied when both strains were compared indicating changes brought about by the inactivation of TCS12. These results are discussed below.

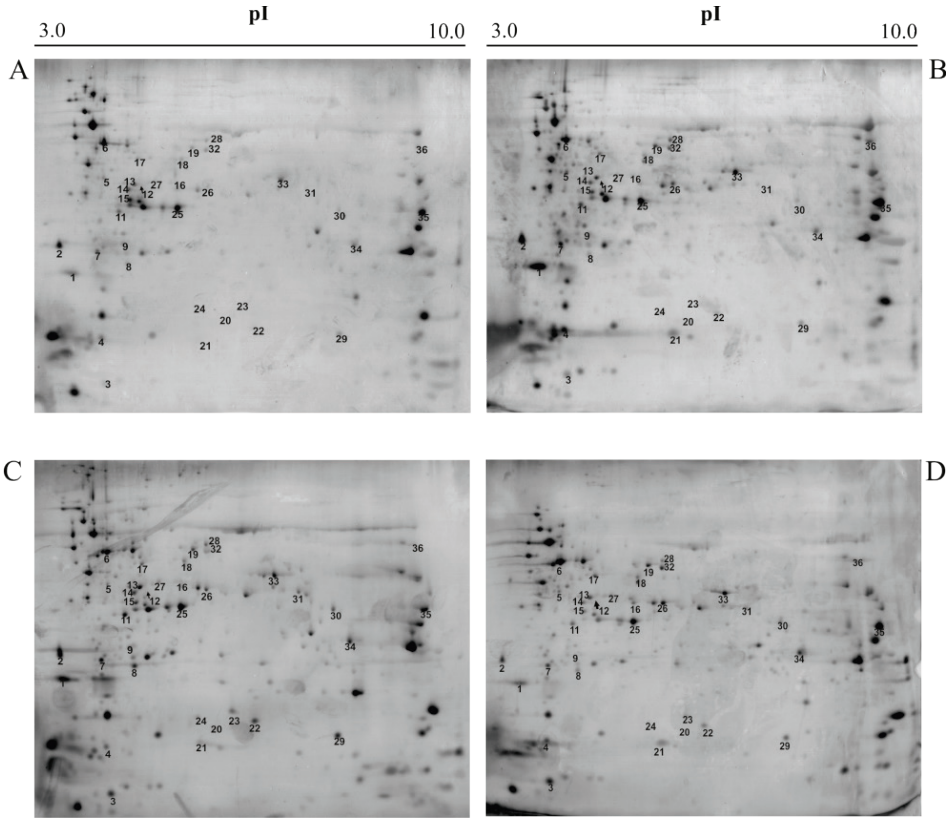


Figure 3. Silver-stained 2D electrophoresis gels of cell membrane fraction proteins extracted from *L. casei* BL23 or ΔRR12 cells diluted in MRS (pH 6.8) or acid MRS (pH 4.4). **A**, BL23 pH 6.8; **B**, ΔRR12 pH 6.8; **C**, BL23 pH 4.4; **D**, ΔRR12 pH 4.4. The figure shows one representative gel for each sample. Spot numbers indicate differentially expressed proteins.

Table 3. Differential spots detected in the proteomic analysis of the membrane protein fractions of *L. casei* BL23 and ΔRR12 mutant cells after an acid challenge at pH 4.4 for 45 minutes.

Spot Nº	Putative function*	Locus tag (gene name)	Ratio†			
			BL23 pH/ BL23 MRS	ΔRR12 pH/ ΔRR12 MRS	ΔRR12 MRS /BL23 MRS	ΔRR12 pH /BL23 pH
Stress response						
LC03	10 kDa chaperonin (groES protein)	LCABL_24210 *(groS)*	**2.68**‡	**4.11**	1.00	1.54
LC22	Universal stress protein, UspA family	LCABL_14120 *(usp6)*	**4.44**	**4.23**	0.64	**0.61**
LC32	Trypsin-like serine protease	LCABL_30080 *(htrA)*	0.74	**4.02**	0.52	**2.81**
Energy metabolism						
LC12	ATP synthase gamma chain	LCABL_13870 *(atpG)*	0	0	0.82	NC§
LC13	Pyruvate dehydrogenase complex, E1 component, alpha subunit	LCABL_15360 *(pdhA)*	**1.57**	**1.55**	1.02	1.01
LC14	ATP synthase gamma chain	LCABL_13870 *(atpG)*	**2.11**	**1.78**	1.01	0.86
LC15	L-lactate dehydrogenase	LCABL_27160 *(ldh)*	**0.69**	**0.52**	1.20	0.92
LC16	Glyceraldehyde 3-phosphate dehydrogenase	LCABL_11300 *(gap-1)*	1.30	**4.18**	0.61	1.97
LC19	Dihydrolipoyl dehydrogenase	LCABL_15390 *(pdhD)*	**2.73**	**3.01**	1.12	1.23
Carbohydrate transport						
LC20	EIIB phosphotransferase system	LCABL_04730 (*PTS-EIIB*)	0.49	NC	0	0
LC25	EIIAB: Phosphotransferase system; glucose transport	LCABL_30340 *(manL)*	**2.46**	1.16	1.24	0.58
Lipid metabolism						
LC04	(3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase	LCABL_23020 *(fabZ)*	0.72	1.46	**2.10**	**4.24**

Table 3. Continuation.

Spot Nº	Putative function*	Locus tag (gene name)	Ratio†			
			BL23 pH/ BL23 MRS	ΔRR12 pH/ ΔRR12 MRS	ΔRR12 MRS /BL23 MRS	ΔRR12 pH /BL23 pH
Amino acid and peptide transport and metabolism						
LC11	Oligopeptide ABC transporter, substrate-binding lipoprotein	LCABL_22460 *(oppA)*	**2.06**	**1.99**	0.97	0.93
LC26	Oligopeptide ABC transporter, ATP-binding subunit	LCABL_22430 *(oppD)*	**2.86**	**2.23**	**2.11**	**1.65**
LC30	Oligopeptide ABC transporter (ATP-binding protein) Oligopeptide ABC transporter, ATP-binding subunit	LCABL_18260 (*oppF*) LCABL_22420 (*oppF*)	**4.57**	**6.32**	0.71	0.98
LC31	Oligopeptide ABC transporter (ATP-binding protein) Oligopeptide ABC transporter, ATP-binding subunit	LCABL_18260 (*oppF*) LCABL_22420 (*oppF*)	NC	**3.67**	NC	0.90
LC33	Oligopeptide ABC transporter, ATP binding protein	LCABL_21090 *(oppD)*	0.72	1.28	0.95	**1.70**
LC17	Glycine betaine/carnitine/choline ABC transporter, ATP-binding protein	LCABL_00660 (*opuA*)	2.19	**3.12**	0.73	1.04
LC08	Glutamine ABC transporter, ATP-binding protein Proline dipeptidase	LCABL_14910 (*glnQ4)* LCABL_18590 (*pepQ-1*)	**5.83**	**5.15**	0.65	0.58
LC27	Proline dipeptidase	LCABL_18590 (*pepQ-1)*	**2.57**	3.92	0.91	1.39
LC18	Glutamine synthetase	LCABL_18680 *(glnA)*	1.41	**3.84**	0.55	**1.51**
LC28	Glutamyl-tRNA synthetase	LCABL_24920 *(gltX)*	**1.73**	1.78	1.55	**1.60**

Table 3. Continuation.

Spot Nº	Putative function*	Locus tag (gene name)	Ratio† BL23 pH/ BL23 MRS	ΔRR12 pH/ ΔRR12 MRS	ΔRR12 MRS /BL23 MRS	ΔRR12 pH /BL23 pH
Uncharacterized transporters						
LC09	ABC-type antimicrobial peptide transport system, ATPase component	LCABL_21680	1.58	1.53	**0.25**	**0.24**
LC35	ABC-type uncharacterized transport system, periplasmic component	LCABL_07680	0.75	1.36	1.90	**3.46**
Transcription and translation						
LC05	DNA-directed RNA polymerase subunit alpha	LCABL_26450 *(rpoA)*	0.86	1.04	1.75	**2.11**
LC01	50S ribosomal protein L4	LCABL_26690 *(rplD)*	1.45	0.76	**2.41**	1.26
LC06	30S Ribosomal protein S1	LCABL_15990 *(rpsA)*	0.70	0.76	**1.97**	**2.13**
LC29	30S Ribosomal protein S5	LCABL_26540 *(rpsE)*	**2.04**	**2.21**	1.04	1.12
Two possible identification with different funtion						
LC07	Glutamine ABC transporter, ATP-binding protein DNA-directed RNA polymerase subunit alpha	LCABL_15660 (*glnQ3*) LCABL_26450 *(rpoA)*	1,36	**1,88**	0,73	1,01
Uncharacterized or unidentified proteins						
LC36		LCABL_22340 *(wze)*	3.34	0.66	**3.66**	0.73
LC02		LCABL_08670	0.85	**0.44**	**1.94**	1.02
LC21	not identified		2.01	0.66	6.31	2.08
LC23	not identified		**2.63**	1.94	0.54	**0.40**
LC24	not identified		0.67	NC	NC	NC
LC34	not identified		**2.21**	**1.99**	1.23	1.11

* Hypothetical function based on the *L. casei* BL23 genome annotation.
† Normalized volume ratios for each protein at the indicated conditions.
‡ Bold numbers: statistically significant difference (P<0.05).
§ Not calculable.

DISCUSSION

In the present work we have performed a comparative analysis of the response to acid stress of *L. casei* BL23 and the derivative strain ΔRR12. Previous studies showed that the pleiotropic phenotype of strain ΔRR12 was mostly due to low expression of *dlt* (Revilla-Guarinos *et al.*, 2013). Notwithstanding, we decided to focus our study on strain ΔRR12 since a Dlt-defective mutant displayed poor growth and poor data reproducibility in some assays (results not shown).

First, the ability to induce a growth phase dependent ATR of strains BL23 and ΔRR12 was determined. This growth phase dependent ATR is naturally induced as pH of the media decreases as a consequence of the accumulation of acid end-products of metabolism (Buchanan and Edelson, 1996). The results obtained showed that *L. casei* BL23 is able to induce a growth phase dependent ATR and that ΔRR12 also induces an ATR although induction was delayed compared to the parental strain (Table 1 and Fig. 1). Furthermore, this experiment showed that strain ΔRR12 reached the same final pH value than strain BL23. This observation indicates that ΔRR12 remains metabolically active but it is not able to increase the biomass of the culture. The ATR in both strains was induced when the pH of the media decreased below 4.5. At this pH a substantial part of lactic acid is undissociated (23% in pure water) and can enter the cell by diffusion (Lambert and Stratford, 1999). LAB maintain a cytoplasm that is more alkaline than the medium, but whose pH decreases as the medium is acidified during growth (Kashket, 1987). We suggest that ATR in *L. casei* is triggered when pH_{in} reaches a critical value as previously proposed for *Lc. lactis* (O'Sullivan and Condon, 1997). These results indicated that inactivation of TCS12 did not impair induction of ATR but led to a premature entry into stationary phase.

Significant differences in the FA content of both strains and their variation in response to pH were observed. Major FAs were palmitic, oleic, vaccenic and dihydrosterculic acids. This result contrast with values previously reported by Broadbent et al. (Broadbent *et al.*, 2010) but the use of a different experimental design and a different strain may account for these discrepancies. In response to acid stress, bacteria change the membrane FA

composition in order to decrease the proton permeability (Fozo *et al.*, 2004; Fozo and Quivey, 2004; Broadbent *et al.*, 2010). These changes usually involve an increase of the concentration of saturated FAs and cyclopropane FAs (Cotter and Hill, 2003; Fozo *et al.*, 2004; Broadbent *et al.*, 2010) although Wu et al. (Wu *et al.*, 2012) observed an increase in unsaturated FA and cyclopropane FAs in *L. casei* Zhang in response to acid stress. In the present study, an increase in cyclopropane FAs and a concomitant decrease of the unsaturated FA content was observed in both strains as pH decreased. A similar trend had been observed in *L. casei* ATCC 334 (Broadbent *et al.*, 2010). However, whereas it was observed a dramatic increase of the content of myristic acid of strain ATCC 344 in response to acid, no significant differences in the content of myristic acid were observed in either strain BL23 or its derivative ΔRR12. Again, different experimental conditions and strain may explain this difference. On the other hand, significant differences in FA content were observed between strains BL23 and ΔRR12 and changes in the FA content in response to decreasing pH also varied (Table 2). This observation correlated with an increase in the abundance of FabZ in ΔRR12 compared to BL23 (Table 3). *L. casei* FabZ is homologous to the *E. faecalis* V583 FabZ1 protein which has been shown to be a dual enzyme that converts β-hydroxyacyl-ACPs to *trans*-2 unsaturated acyl-ACPs and isomerizes them into *cis*-3-decenoyl-ACPs, the key step of the classical anaerobic unsaturated fatty acid biosynthetic pathway (Wang and Cronan, 2004). These results indicate that the inactivation of TCS12 affected the membrane FA content although additional data are required to explain the consequences of this fact.

To further investigate the response of *L. casei* BL23 and ΔRR12 to acidic pH a proteomic approach was used to identify differentially produced membrane associated proteins from cells exposed to pH 4.4 for 45 minutes. This pH was chosen because ΔRR12 mutant prematurely enters in stationary phase around this value (Fig. 1) and significant differences in ATR induction between the wild-type and the mutant were observed when the pH of the media was between 4.5 and 4 (Fig 1 and Table 1). An exposition time of 45 minutes was chosen. Hamilton and colleagues (Hamilton and Svensäter, 1998) reported that most of the proteins induced in response to acid shock in *L. casei* 151 were expressed between 30 and 60 minutes after shock. Since our

previous data suggested that TCS12 inactivation mostly affected the cell envelope (Alcántara *et al.*, 2011; Revilla-Guarinos *et al.*, 2013) and changes in cytoplasmic proteins in response to low pH in *L. casei* had already been characterized (Wu *et al.*, 2011), we decided to analyze in detail proteins associated to the cell membrane.

Acid stress led to an increased abundance of two stress proteins in both strains, GroES and UspA (Table 3). Induction of stress proteins was also observed in *L. casei* Zhang (Wu *et al.*, 2011; Wu *et al.*, 2012). In contrast, Broadbent et al. (Broadbent *et al.*, 2010) observed a decrease in the expression of stress response proteins after acid adaptation. Furthermore, the trypsin-like serine protease HtrA was specifically induced in response to pH in the mutant strain ΔRR12 (Table 3). HtrA is predicted to be located at the cell membrane and it has been shown to participate in the degradation of abnormal exported proteins in *Lc. lactis* (Poquet *et al.*, 2000) and streptococci (Tsui *et al.*, 2011; Cassone *et al.*, 2012). The induction of this protein had been previously observed in *L. casei* BL23 in response to p-coumaric acid (Rivas-Sendra *et al.*, 2011) where it was proposed that p-coumaric acid disturbed protein structure at the cell envelope. This result suggests that low pH damaged cell envelope proteins to a greater extent in strain ΔRR12 than in the parental strain.

Changes were also observed in both strains in the abundance of proteins participating in energy production. An increase of the α subunit and the dihydrolipoyl dehydrogenase of the pyruvate dehydrogenase (PDH) complex and a decrease of L-lactate dehydrogenase (Ldh) was observed. The increase in PDH and decrease in Ldh abundances after acid exposure may suggest that in *L.casei* BL23 there is an increase in the metabolic pathway from pyruvate to acetyl CoA by means of the PDH complex, and a reduction in the flux from pyruvate to L-lactate, in response to acid. Similar results had been previously described in *Lactobacillus rhamnosus* GG where Ldh was found to be less abundant and pyruvate dehydrogenase complex E2 component (PdhC) more abundant when grown at pH 4.8 relative to pH 5.8, suggesting a shift of pyruvate metabolism towards the formation of acetyl-CoA (Koponen *et al.*, 2012). Furthermore, the same change in pyruvate metabolism was observed in *Lactobacillus bulgaricus* during adaptation to pH 4.9 (Fernández *et al.*, 2008).

However, it must be considered that the result presented here was obtained from the analysis of membrane-associated proteins and Ldh has been previously reported in the cell envelope of *L. casei* (Nezhad *et al.*, 2012) and therefore it may reflect a redistribution of Ldh between cytoplasm and cell envelope rather than a net decrease of the Ldh content of the cell.

A 4.18 fold increased abundance of glyceraldehyde 3-phosphate dehydrogenase (Gap) was observed in strain ΔRR12 in response to acid (Table 3). Association of Gap to the cell envelope has been previously reported in other bacteria (Pancholi and Fischetti, 1992; Ruiz *et al.*, 2009) including lactobacilli (Antikainen *et al.*, 2007; Nezhad *et al.*, 2012; Kinoshita *et al.*, 2013). Interestingly, Antikainen et al. (Antikainen *et al.*, 2007) observed that Gap is associated to the cell envelope in *Lactobacillus crispatus* at acidic pH whereas at neutral pH is rapidly released to the media and that Gap interacts with negatively charged LTAs. The increased abundance of Gap in strain ΔRR12 may therefore relate to low D-alanylation of LTAs in this strain compared to the parental strain BL23 (Revilla-Guarinos *et al.*, 2013). However, an increased association of Gap with the membrane within the cell cannot be ruled out with the data currently available.

A controversial result was obtained for the subunit γ of the F_0F_1-ATPase after exposure to acid since it was identified in two spots. While spot LC12 disappeared after pH exposure in both strains, spot LC14 showed a significant increase in response to pH in both strains. This result may indicate a post-translational modification of this subunit in response to pH. The F_0F_1-ATPase plays a crucial role in acid tolerance in lactobacilli (Corcoran *et al.*, 2008) but regulation of its activity in these organisms is still poorly understood. Regulation of the activity of chloroplast ATPase by the redox state of two Cys residues in subunit γ has been reported (Arana and Vallejos, 1982) although the regulatory domain encompassing these cysteines is absent in bacterial γ subunits. As far as we know, post-translational modifications of γ subunit have not been reported in bacterial F_0F_1-ATPases.

Adaptation to low pH also brought about changes in the abundance of proteins involved in peptide and amino acid metabolism as well as putative

amino acid and peptide transporters (Table 3). In particular, our results showed that several subunits of oligopeptide ABC transporters (Opp) were more abundant after acid exposure, both in the wild type and the mutant strain (Table 3). *L. casei* BL23 encodes three putative Opp systems (Mazé *et al.*, 2010) but most spots identified belonged to the Opp system encoded by the gene cluster LCABL_22420-LCABL_22460. This result agrees with that reported by Broadbent et al. (Broadbent *et al.*, 2010) who observed an increased expression of the homologous Opp system of *L. casei* ATCC334 (LSEI_2061-LSEI_2065) after 20 minutes of acid adaptation at pH 4.5. In contrast, a decrease of the homologous OppA subunit was observed in *L. rhamnosus* GG (Koponen *et al.*, 2012).

Glutamine synthetase (*glnA*) was significantly more abundant in the mutant strain in response to acid exposure relative to reference conditions (ratio 3.84) and it was also more abundant in the mutant relative to the wild-type in response to acid (ratio 1.51). Location of GlnA on the cell envelope has been previously reported in bifidobacteria (Candela *et al.*, 2007; Ruiz *et al.*, 2009) and *L. crispatus* (Kainulainen *et al.*, 2012). As previously described for Gap (see above) binding of GlnA to the cell envelope preferentially occurred at low pH (Kainulainen *et al.*, 2012) thus suggesting that GlnA also binds to LTAs. However, and as indicated for Gap, an increased association with the membrane inside the cell cannot be ruled out.

The ATPase component of a putative ABC antimicrobial peptide transport system (LCABL_21680) was less abundant in the mutant relative to the wild type both in reference conditions and in response to acid. This ABC transporter is the Orphan ABC transporter (OrABC) that we previously described to be under transcriptional control of TCS12 (Revilla-Guarinos *et al.*, 2013) although its functional role has not been ascertained. Interestingly, Wu et al reported that this ABC transporter was 3.3 fold induced in the *L. casei* Zhang acid resistant strain relative to the wild type, after acid challenge (Wu *et al.*, 2012). A subunit of a putative sugar ABC transporter was more abundant in ΔRR12 in acidic conditions (Table 3) although its functional role has not been established.

A number of ribosomal proteins also showed significant differences in abundance in response to acid (Table 3). Association of ribosomal proteins with the cell envelope has been previously observed in a number of studies (Wilkins *et al.*, 2003; Ruiz *et al.*, 2009; Sánchez *et al.*, 2009; Koskenniemi *et al.*, 2011) although their role at this location has not been determined. An increase in the abundance of the ribosomal protein S5 (RpsE) was detected in both strains (Table 3). Interestingly, increased abundance of RpsE in the cell envelope of *L. rhamnosus* in response to bile had been previously observed (Koskenniemi *et al.*, 2011). These results may suggest that RpsE play a role in the response against cell envelope stress in these organisms.

In summary, the results reported here showed that *L. casei* BL23 and ΔRR12 induce a growth phase dependent ATR response although the latter prematurely enters into stationary phase whereas the wild-type strain keeps growing. The analysis of CMFAs and protein content of the cell envelope revealed significant differences between both strains that correlate with the differences in sensitivity to cationic antimicrobial peptides previously reported (Alcántara *et al.*, 2011; Revilla-Guarinos *et al.*, 2013). In particular, the greater abundance of cytoplasmic proteins such as Gap and GlnA in the cell envelope of the mutant strain may indicate a greater permeability of the cell membrane in ΔRR12. The mechanism by which these proteins are exported to the cell wall has not been clearly established. However, evidence has been reported pointing to a link between increased cell membrane permeability and efflux of cytoplasmic proteins in *Lactobacillus plantarum* (Saad *et al.*, 2009) and recently a similar result was reported for *L. crispatus* (Kainulainen *et al.*, 2012). Therefore, the results presented here suggest that D-alanylation of TAs has a remarkable influence on the functioning of the cell membrane of *L. casei* although further research will be required to elucidate this point. Furthermore, differences in acid tolerance between *L. casei* BL23 and its derivative strain ΔRR12 are possibly related to differences in surface physiology.

ACKNOWLEDGEMENTS

This work was financed by funds from the former Spanish Ministry of Science and Innovation (AGL2007-60975 and AGL2010-15679) and Generalitat Valenciana (ACOMP2012/137). A. Revilla-Guarinos was the recipient of a research fellowship from the Instituto Danone.
We thank Amalia Blasco for technical assistance.

SUPPLEMENTAL MATERIAL

Table S1. Characteristics of the identified protein spots differentially expressed of *L. casei* BL23 and ΔRR12 mutant cells after an acid challenge at pH 4.4 for 45 minutes.

Spot Nº	Locus tag	Mass (Da)[1]	pI[2]	Nº aa[3]	Coverage (%)[4]	Nº matched peptides[5]	COG	Normalized volume BL23 control MRS ($\times10^{-04}$)[6]	Normalized volume BL23 pH ($\times10^{-04}$)	Normalized volume ΔRR12 control MRS ($\times10^{-04}$)[6]	Normalized volume ΔRR12 pH ($\times10^{-04}$)
LC01	LCABL_26690	22428,17969	9.88	207	22	4	**COG88**	6,69±1,69	9,71±7,51	16,12±4,53	12,21±8,02
LC02	LCABL_08670	18729,93945	4.22	175	24	4	h.p.	6,01±2,06	5,09±1,07	11,70±2,70	5,19±1,76
LC03	LCABL_24210	10033,29004	4.93	93	37	2	**COG234**	1,80±1,40	4,83±1,12	1,81±1,33	7,43±2,66
LC04	LCABL_23020	15591,16992	4,91	145	40	4	**COG764**	2,41±1,86	1,74±0,42	5,06±2,36	7,39±5,59
LC05	LCABL_26450	34736,98047	5.01	312	45	16	**COG202**	0,61±0,38	0,53±0,30	1,07±0,59	1,11±0,36
LC06	LCABL_15990	47620,69922	5.04	439	38	10	**COG539**	7,94±2,89	5,58±2,25	15,66±8,08	11,90±3,30
LC07	LCABL_15660 LCABL_26450	27294,0605 34736,9805	4.98 5.01	248 312	44 23	8 7	**COG1126** **COG202**	2,20±0,90	2,99±1,34	1,61±0,60	3,03±0,92
LC08	LCABL_14910 LCABL_18590	22806,94922 38886,87891	5.23 5.39	212 355	57 58	7 14	**COG1126** **COG6**	0,61±0,28	3,57±1,27	0,40±0,23	2,06±0,86
LC09	LCABL_21680	27109	5.33	252	69	i.p.s.	**COG1136**	0,94±0,39	1,49±0,49	0,23±0,09	0,36±0,11
LC11	LCABL_22460	59487	9.5	540	42	19	**COG4166**	1,95±0,37	4,02±0,61	1,88±0,48	3,74±1,03
LC12	LCABL_13870	33688,01953	5.15	308	43	16	**COG224**	1,04±0,35	(-)[7]	0,85±0,03	(-)
LC13	LCABL_15360	40787,67188	5.29	370	58	14	**COG1071**	1,99±0,43	3,12±0,75	2,03±0,45	3,16±0,86
LC14	LCABL_13870	33688,01953	5.15	308	43	16	**COG224**	1,26±0,52	2,66±0,78	1,28±0,74	2,28±0,69
LC15	LCABL_27160	35508,44922	5.24	326	44	14	**COG39**	2,95±0,83	2,02±0,40	3,53±1,17	1,85±0,33
LC16	LCABL_11300	36912	5.68	340	49	i.p.s.	**COG57**	0,38±0,15	0,49±0,21	0,23±0,08	0,97±0,71

Table S1. Continuation.

Spot Nº	Locus tag	Mass (Da)[1]	pI[2]	Nº aa[3]	Coverage (%)[4]	Nº matched peptides[5]	COG	Normalized volume BL23 control MRS ($\times10^{-04}$)[6]	Normalized volume BL23 pH ($\times10^{-04}$)	Normalized volume ΔRR12 control MRS ($\times10^{-04}$)[6]	Normalized volume ΔRR12 pH ($\times10^{-04}$)
LC17	LCABL_00660	46570	5.22	417	60	19	**COG1125**	0,63±0,24	1,37±0,82	0,46±0,17	1,43±0,62
LC18	LCABL_18680	50364	5.58	446	58	18	**COG174**	0,63±0,48	0,88±0,16	0,35±0,11	1,34±0,34
LC19	LCABL_15390	49241	5.79	467	18	i.p.s.	**COG1249**	0,66±0,25	1,81±0,59	0,74±0,44	2,23±0,67
LC20	LCABL_04730	18663	5.31	168	51	i.p.s.	**COG3444**	0,32±0,30	0,16±0,09	(-)	(-)
LC21	not identified[8]							0,84±0,58	1,70±1,27	5,33±4,97	3,52±2,30
LC22	LCABL_14120	17974	6.13	165	73	12	**COG589**	0,66±0,63	2,93±0,60	0,42±0,41	1,79±0,32
LC23	not identified							0,70±0,72	1,84±0,60	0,38±0,26	0,73±0,53
LC24	not identified							0,37±0,01	0,24±0,16	(-)	(-)
LC25	LCABL_30340	35296	5.48	324	58	i.p.s.	**COG3444**	7,38±1,46	18,17±9,79	9,14±2,32	10,61±5,37
LC26	LCABL_22430	38285	5.78	350	54	16	**COG444**	0,67±0,16	1,92±0,72	1,42±0,58	3,16±1,05
LC27	LCABL_18590	38887	5.39	355	52	12	**COG6**	0,24±0,05	0,61±0,19	0,21±0,10	0,84±0,36
LC28	LCABL_24920	56835	5.73	497	45	i.p.s.	**COG8**	0,51±0,20	0,88±0,35	0,79±0,53	1,42±0,40
LC29	LCABL_26540	17524	6.85	167	54	9	**COG98**	2,17±0,58	4,43±0,36	2,25±1,12	4,97±0,95
LC30	LCABL_18260	36324,55859	6.88	317	67	24	**COG4608**	0,37±0,10	1,71±1,10	0,27±0,17	1,68±0,43
	LCABL_22420	36290,87891	6.32	325	17	3	**COG4608**				

Table S1. Continuation.

Spot Nº	Locus tag	Mass (Da)[1]	pI[2]	Nº aa[3]	Coverage (%)[4]	Nº matched peptides[5]	COG	Normalized volume BL23 control MRS ($\times10^{-04}$)[6]	Normalized volume BL23 pH ($\times10^{-04}$)	Normalized volume ΔRR12 control MRS ($\times10^{-04}$)[6]	Normalized volume ΔRR12 pH ($\times10^{-04}$)
LC31	LCABL_18260	36324,55859	6.88	317	47	15	**COG4608**	(-)	1,32±0,78	0,32±0,08	1,18±0,32
	LCABL_22420	36290,87891	6.32	325	14	3	**COG4608**				
LC32	LCABL_30080	44924	5.99	442	34	i.p.s.	**COG265**	1,31±0,57	0,97±0,62	0,68±0,40	2,73±1,44
LC33	LCABL_21090	39687,73828	6.23	362	48	14	**COG444**	4,44±2,05	3,18±0,82	4,22±1,34	5,39±1,48
LC34	not identified							1,58±0,57	3,49±0,27	1,95±0,46	3,89±1,73
LC35	LCABL_07680	34099,42969	9.71	322	24	8	**COG2984**	5,46±3,82	4,07±1,52	10,37±5,98	14,11±4,15
LC36	LCABL_22340	27588	7.78	252	52	12	**COG489**	0,48±0,23	1,59±1,23	1,74±1,04	1,15±0,42

[1]Theoretical molecular weight.
[2] Theoretical isoelectric point.
[3] Number of amino acid residues.
[4] Percentage of amino acid coverage (peptides observed/theoretical value from sequence data).
[5] Number of tryptic peptides observed contributing to the percentage of amino acid coverage.
[6] Normalized relative volumes, expressed as a percentage of total valid spots. Values are means ± standard deviations; $n \geq 3$.
[7] (-) Spot not detected.
[8] Spots that did not render reliable mass spectra data.
h.p. Hypothetical protein.
i.p.s. Identified in previous studies

REFERENCES

Alcántara, C., A. Revilla-Guarinos*, et al.* (2011). "Influence of two-component signal transduction systems of *Lactobacillus casei* BL23 on tolerance to stress conditions." Appl. Environ. Microbiol. **77**(4): 1516-1519.

Alcántara, C. and M. Zúñiga (2012). "Proteomic and transcriptomic analysis of the response to bile stress of *Lactobacillus casei* BL23." Microbiology **158**(Pt 5): 1206-1218.

Antikainen, J., V. Kuparinen*, et al.* (2007). "pH-dependent association of enolase and glyceraldehyde-3-phosphate dehydrogenase of *Lactobacillus crispatus* with the cell wall and lipoteichoic acids." J. Bacteriol. **189**(12): 4539-4543.

Arana, J. L. and R. H. Vallejos (1982). "Involvement of sulfhydryl-groups in the activation mechanism of the ATPase activity of chloroplast coupling factor-I." J. Biol. Chem. **257**(3): 1125-1127.

Azcarate-Peril, M. A., O. McAuliffe*, et al.* (2005). "Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in Lactobacillus acidophilus." Appl. Environ. Microbiol. **71**(10): 5794-5804.

Booth, I. R. (1985). "Regulation of cytoplasmic pH in bacteria." Microbiol. Rev. **49**(4): 359-378.

Boyd, D. A., D. G. Cvitkovitch*, et al.* (2000). "Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity." J. Bacteriol. **182**(21): 6055-6065.

Broadbent, J. R., R. L. Larsen*, et al.* (2010). "Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress." J. Bacteriol. **192**(9): 2445-2458.

Buchanan, R. L. and S. G. Edelson (1996). "Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells." Appl. Environ. Microbiol. **62**(11): 4009-4013.

Candela, M., S. Bergmann*, et al.* (2007). "Binding of human plasminogen to *Bifidobacterium*." J. Bacteriol. **189**(16): 5929-5936.

Cassone, M., A. L. Gagne*, et al.* (2012). "The HtrA protease from *Streptococcus pneumoniae* digests both denatured proteins and the competence-stimulating peptide." J. Biol. Chem. **287**(46): 38449-38459.

Corcoran, B. M., C. Stanton*, et al.* (2008). "Life under stress: the probiotic stress response and how it may be manipulated." Curr. Pharm. Des. **14**(14): 1382-1399.

Cotter, P. D. and C. Hill (2003). "Surviving the acid test: responses of gram-positive bacteria to low pH." Microbiol. Mol. Biol. Rev. **67**(3): 429-453, table of contents.

De Angelis, M., L. Bini*, et al.* (2001). "The acid-stress response in *Lactobacillus sanfranciscensis* CB1." Microbiology **147**(Pt 7): 1863-1873.

De Angelis, M. and M. Gobbetti (2004). "Environmental stress responses in *Lactobacillus*: a review." PROTEOMICS **4**(1): 106-122.

de Vrese, M. and J. Schrezenmeir (2008). "Probiotics, prebiotics, and synbiotics." Adv. Biochem. Eng. Biotechnol. **111**: 1-66.

Fernández, A., J. Ogawa*, et al.* (2008). "Rerouting of pyruvate metabolism during acid adaptation in *Lactobacillus bulgaricus*." Proteomics **8**(15): 3154-3163.

Fozo, E. M., J. K. Kajfasz*, et al.* (2004). "Low pH-induced membrane fatty acid alterations in oral bacteria." FEMS Microbiol. Lett. **238**(2): 291-295.

Fozo, E. M. and R. G. Quivey, Jr. (2004). "Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments." Appl. Environ. Microbiol. **70**(2): 929-936.

Frees, D., F. K. Vogensen*, et al.* (2003). "Identification of proteins induced at low pH in *Lactococcus lactis*." Int. J. Food Microbiol. **87**(3): 293-300.

Goodson, M. and R. J. Rowbury (1989). "Habituation to normally lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value." Lett. Appl. Microbiol. **8**(2): 77-79.

Hamilton, I. R. and G. Svensäter (1998). "Acid-regulated proteins induced by *Streptococcus mutans* and other oral bacteria during acid shock." Oral Microbiol. Immunol. **13**(5): 292-300.

Han, H., C. Liu*, et al.* (2012). "The two-component system Ihk/Irr contributes to the virulence of *Streptococcus suis* serotype 2 strain 05ZYH33 through alteration of the bacterial cell metabolism." Microbiology **158**(Pt 7): 1852-1866.

Hutkins, R. W. and N. L. Nannen (1993). "pH homeostasis in lactic acid bacteria." J. Dairy Sci. **76**(8): 2354-2365.

Ichikawa, M., M. Minami*, et al.* (2011). "Analysis of two-component sensor proteins involved in the response to acid stimuli in *Streptococcus pyogenes*." Microbiology **157**(Pt 11): 3187-3194.

Kainulainen, V., V. Loimaranta*, et al.* (2012). "Glutamine synthetase and glucose-6-phosphate isomerase are adhesive moonlighting proteins of *Lactobacillus crispatus* released by epithelial cathelicidin LL-37." J. Bacteriol. **194**(10): 2509-2519.

Kashket, E. R. (1987). "Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance." FEMS Microbiol. Rev. **46**(3): 233-244.

Kinoshita, H., S. Imoto*, et al.* (2013). "Proposal of screening method for intestinal mucus adhesive lactobacilli using the enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH)." Anim. Sci. J. **84**(2): 150-158.

Kleerebezem, M. and E. E. Vaughan (2009). "Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity." Annu. Rev. Microbiol. **63**: 269-290.

Koponen, J., K. Laakso*, et al.* (2012). "Effect of acid stress on protein expression and phosphorylation in *Lactobacillus rhamnosus* GG." J. Proteomics **75**(4): 1357-1374.

Koskenniemi, K., K. Laakso*, et al.* (2011). "Proteomics and transcriptomics characterization of bile stress response in probiotic *Lactobacillus rhamnosus* GG." Mol Cell Proteomics **10**(2): M110 002741.

Lambert, R. J. and M. Stratford (1999). "Weak-acid preservatives: modelling microbial inhibition and response." J. Appl. Microbiol. **86**(1): 157-164.

Lim, E. M., S. D. Ehrlich*, et al.* (2000). "Identification of stress-inducible proteins in *Lactobacillus delbrueckii* subsp. *bulgaricus*." Electrophoresis **21**(12): 2557-2561.

Mazé, A., G. Böel*, et al.* (2010). "Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23." J. Bacteriol. **192**(10): 2647-2648.

Morel-Deville, F., F. Fauvel*, et al.* (1998). "Two-component signal-transducing systems involved in stress responses and vancomycin susceptibility in *Lactobacillus sakei*." Microbiology **144**(10): 2873-2883.

Nezhad, M. H., M. Knight*, et al.* (2012). "Evidence of changes in cell surface proteins during growth of *Lactobacillus casei* under acidic conditions." Food Sci. Biotechnol. **21**(1): 253-260.

O'Connell-Motherway, M., D. van Sinderen*, et al.* (2000). "Six putative two-component regulatory systems isolated from *Lactococcus lactis* subsp. *cremoris* MG1363." Microbiology **146**(4): 935-947.

O'Sullivan, E. and S. Condon (1997). "Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis*." Appl. Environ. Microbiol. **63**(11): 4210-4215.

Pancholi, V. and V. A. Fischetti (1992). "A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity." J. Exp. Med. **176**(2): 415-426.

Poquet, I., V. Saint*, et al.* (2000). "HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing." Mol Microbiol **35**(5): 1042-1051.

Rallu, F., A. Gruss*, et al.* (2000). "Acid- and multistress-resistant mutants of *Lactococcus lactis*: identification of intracellular stress signals." Mol. Microbiol. **35**(3): 517-528.

Revilla-Guarinos, A., S. Gebhard*, et al.* (2013). "Characterization of a regulatory network of peptide antibiotic detoxification modules in *Lactobacillus casei* BL23." Appl. Environ. Microbiol. **79**(10): 3160-3170.

Rivas-Sendra, A., J. M. Landete*, et al.* (2011). "Response of *Lactobacillus casei* BL23 to phenolic compounds." J Appl Microbiol **111**(6): 1473-1481.

Rozès, N., S. Garbay*, et al.* (1993). "A rapid method for the determination of bacterial fatty acid composition." Lett. Appl. Microbiol. **17**(3): 126-131.

Ruiz, L., Y. Couté*, et al.* (2009). "The cell-envelope proteome of *Bifidobacterium longum* in an in vitro bile environment." Microbiology **155**(Pt 3): 957-967.

Saad, N., M. Urdaci*, et al.* (2009). "*Lactobacillus plantarum* 299v surface-bound GAPDH: a new insight into enzyme cell walls location." J. Microbiol. Biotechnol. **19**(12): 1635-1643.

Sánchez, B., P. Bressollier*, et al.* (2009). "Identification of surface-associated proteins in the probiotic bacterium *Lactobacillus rhamnosus* GG." Int. Dairy J. **19**(2): 85-88.

Tsui, H.-C. T., S. K. Keen*, et al.* (2011). "Dynamic distribution of the SecA and SecY translocase subunits and septal localization of the HtrA surface chaperone/protease during *Streptococcus pneumoniae* D39 cell division." Mbio **2**(5): e0020211.

van de Guchte, M., P. Serror*, et al.* (2002). "Stress responses in lactic acid bacteria." Antonie Van Leeuwenhoek **82**(1-4): 187-216.

Vélez, M. P., T. L. A. Verhoeven*, et al.* (2007). "Functional analysis of D-alanylation of lipoteichoic acid in the probiotic strain *Lactobacillus rhamnosus* GG." Appl. Environ. Microbiol. **73**(11): 3595-3604.

Walter, J. and R. Ley (2011). "The human gut microbiome: ecology and recent evolutionary changes." Annu. Rev. Microbiol. **65**: 411-429.

Wang, H. and J. E. Cronan (2004). "Functional replacement of the FabA and FabB proteins of *Escherichia coli* fatty acid synthesis by *Enterococcus faecalis* FabZ and FabF homologues." J. Biol. Chem. **279**(33): 34489-34495.

Wilkins, J. C., D. Beighton*, et al.* (2003). "Effect of acidic pH on expression of surface-associated proteins of *Streptococcus oralis*." Appl. Environ. Microbiol. **69**(9): 5290-5296.

Wolf, C., F. Hochgräfe*, et al.* (2008). "Proteomic analysis of antioxidant strategies of *Staphylococcus aureus*: diverse responses to different oxidants." PROTEOMICS **8**(15): 3139-3153.

Wu, C., J. Zhang*, et al.* (2012). "A combined physiological and proteomic approach to reveal lactic-acid-induced alterations in *Lactobacillus casei* Zhang and its mutant with enhanced lactic acid tolerance." Appl. Microbiol. Biotechnol. **93**(2): 707-722.

Wu, C., J. Zhang*, et al.* (2012). "*Lactobacillus casei* combats acid stress by maintaining cell membrane functionality." J. Ind. Microbiol. Biotechnol. **39**(7): 1031-1039.

Wu, R., W. Zhang*, et al.* (2011). "Proteomic analysis of responses of a new probiotic bacterium *Lactobacillus casei* Zhang to low acid stress." Int J Food Microbiol **147**(3): 181-187.

GENERAL DISCUSSION & CONCLUSIONS

Los puntos de vista diferentes amplían el horizonte.

Anónimo

GENERAL DISCUSSION

The ability to sense environmental stresses and to give an appropriate response is crucial for the survival of microorganisms in the challenging environments they inhabit. Although the Gram-positive and Gram-negative cell envelope architectures considerably differ, common regulatory themes can be found orchestrating the corresponding cell envelope stress responses (CESR). The mechanisms involved in the CESR have been extensively studied in model organisms like *E. coli* and *B. subtilis*, where TCS and extracytoplasmic function (ECF) σ factors are the main players (Heimann, 2002; Ruiz and Silhavy, 2005; Rowley *et al.*, 2006; Jordan *et al.*, 2008).

The CESR is of special interest in the case of probiotic bacteria since they must survive not only the transit through the gastrointestinal tract but also industrial production processes. This has driven the study of the stress response of lactic acid bacteria (van de Guchte *et al.*, 2002; Spano and Massa, 2006; Bron and Kleerebezem, 2011; Wu *et al.*, 2013) although only a few studies have focused on *Lactobacillus casei*, the strain subject of the present study (Neuhaus, 1985; De Angelis and Gobbetti, 2004; Piuri *et al.*, 2005; Broadbent *et al.*, 2010; Li *et al.*, 2010; Rivas-Sendra *et al.*, 2011; Wu *et al.*, 2012; Palomino *et al.*, 2013). Nevertheless, the mechanisms regulating the stress response in lactic acid bacteria are little known. Particularly the mechanisms regulating the stress response in *Lactobacillus* have not been characterized so far. The absence of ECF σ factors in *Lactobacillus* would presumably determine a great difference between the CESR of these organisms with respect to the model Gram-positive *B. subtilis,* where ECF σ factors together with TCS play critical roles, as previously mentioned. However, studies on TCS in *Lactobacillus* are limited (Azcarate-Peril *et al.*, 2005; Pfeiler *et al.*, 2007; Landete *et al.*, 2010; Rivas-Sendra *et al.*, 2011).

The general objective of this PhD thesis was to investigate the role of TCS in the stress response of *Lactobacillus casei* BL23, which is the reference strain in the "Laboratorio de Bacterias Lácticas y Probióticos" where this work was carried out. The availability of its genomic sequence (Mazé *et al.*, 2010) and developed molecular techniques makes from BL23 an ideal model strain for the *Lactobacillus casei* species.

In chapter 1 part I, “Influence of two-component signal transduction systems of *Lactobacillus casei* BL23 on tolerance to stress conditions” and part II, *“Involvement of Two-Component Signal Transduction Systems of Lactobacillus casei BL23 on its general physiology”* we identified 17 TCS present in the genome of *L. casei* BL23; viable mutants in the RRs of all of them were obtained and these mutants were subjected to a variety of stress conditions to gain insight into the role of TCS in the stress response of *L. casei*.

The results obtained evidenced that several TCS play a major role in the regulation of *L. casei* physiology. In this way, inactivation of TCS04 and TCS11 resulted in decreased growth rates in reference conditions and inactivation of TCS12 in decreased maximum cell density. Furthermore, the TCS11 mutant displayed a faster acidification rate of milk than the wild-type strain. However, TCS04 and TCS11 mutants did not display a comparatively lower ability to respond to stress conditions. This result suggested that TCS04 and TCS11 possibly play a relevant role in the regulation of growth in *L. casei* although not specifically in stress response.

On the other hand, our results showed that inactivation of systems TC01, TC06, and TC12 led to major growth defects under the stress conditions assayed. Furthermore, inactivation of TCS01, TCS04, TCS06, TCS09, TCS10, TCS11, TCS12, TCS15, TCS16 and TCS17, led to differences in antibiotic sensitivities relative to the wild type. This result indicated that TCS01, TCS06 and TCS12 were involved in the cell envelope stress response.

Furthermore, the results presented in Chapter 1 highlighted that functional predictions based on orthology/homology for TCS must be taken with great caution in *Lactobacillus*, since homologous TCS seem to have different physiological roles despite close phylogenetic relationships between different *Lactobacillus* species.

Subsequent studies were focused on TCS12 and the paralogous TCS09. TCS09 and TCS12 belong to the BceRS-like TCS group. They possess intramembrane sensing HKs and are located next to genes encoding ABC transporters (ABC09 and ABC12) of the peptide-7 exporter family (Pep7E). A third Pep7E ABC transporter not genetically associated with any TCS (orphan

ABC, OrABC) is also encoded in the genome of *L. casei* BL23. This group of TCS has been shown to be involved in the response against AMPs in other microorganisms (Ohki *et al.*, 2003; Collins *et al.*, 2010; Ouyang *et al.*, 2010; Staroń *et al.*, 2011) and characteristically they form functional units together with their cognate ABC transporters (Coumes-Florens *et al.*, 2011; Dintner *et al.*, 2011; Hiron *et al.*, 2011).

In Chapter 2, "Characterization of a regulatory network of peptide antibiotic detoxification modules in *Lactobacillus casei* BL23", the involvement of modules TCS09/ABC09 and TCS12/ABC12 in AMP resistance was addressed and experiments were done to determine if TCS09/ABC09 and TCS12/ABC12 work as functional units as previously described for other BceRS/BceAB homologous systems. In order to ascertain these points, a collection of mutant strains defective in the RR of TCS09 and TCS12 (ΔRR09 and ΔRR12, respectively) and the permease subunits of the ABC transporters 09 and 12 (insertional mutants P09 and P12, respectively) were characterized. The results obtained showed that the mutant strains defective in the RR or the ABC permease of the same module displayed identical AMPs sensitivity phenotypes and identical transcriptional profiles. This result indicated that each TCS constitutes a functional unit with its corresponding ABC transporter as it had been previously described for other systems of this class.

In the case of module TCS09/ABC09, Inactivation of either RR09 or permease 09 led to higher sensitivity to bacitracin, nisin, plectasin and subtilin than that observed in the wild-type strain. The study of the BL23 transcriptional response to nisin showed that the genes coding for ABC09 but not RR09, are induced in *L.casei* BL23 in response to nisin in a concentration dependent manner. This induction was lost in the mutant strains ΔRR09 and P09. Together our results indicated that module 09 constitutes a stand-along AMP detoxification system and a model was proposed where the target AMP (nisin) is sensed by ABC09 and activates HK09 which in turn transfers the signal to RR09 resulting in the induction of the expression of the ABC09 that confers the resistance (Summarized in Fig. 1 of Chapter 2).

In the case of module TCS12/ABC12, inactivation of either RR12 or permease 12 resulted in higher sensitivity to bacitracin, nisin, mersacidin, plectasin, subtilin and vancomycin. Transcriptional profile results of BL23 showed that small changes in response to nisin were detected in the transcript levels of the genes coding for ABC transporter 12 as well as the RR12 gene. Furthermore, transcriptional profiles of module 12 mutants showed that module 12 regulated the expression of OrABC but not the expression of its cognate ABC12. Together with previous evidences that showed that mutant ΔRR12 was sensitive to environmental stresses like presence of bile, acidic pH and high temperature (Chapter 1), these results prompted the question whether TCS12 could also regulate the expression of genes controlling other cellular functions. Thus, a motif based search of the *L. casei* BL23 genome based on the consensus binding sequence for BceR-like response regulators previously described (Dintner *et al.*, 2011) was carried out in order to identify putative promoters under control of TCS09 or TCS12. Four putative BceR-like target promoters were found in *L. c*asei BL23: two upstream of genes encoding ATPase09 and OrATPase (genes already determined to be under transcriptional control of RR09 and RR12, respectively), and two target promoters upstream of genes LCABL_08540 (a small gene upstream of the *dlt*-operon) and LCABL_24490 (renamed *mprF* in this study because it encodes a putative protein significantly similar to characterized lysylphosphatidylglycerol synthetases). The analysis of the transcriptional levels of *dltA* and *mprF* in *L. casei* BL23 and all derived mutant strains suggested firstly, that module 12 regulates the basal expression of *dlt* operon and *mprF* gene in *L. casei* BL23; secondly, that *L. casei* must harbor an additional regulatory system to control their expression. The phenotypical characterization of a *dltA* mutant demonstrated that the pleiotropic phenotype of module 12 mutants was mostly due to low expression of the Dlt system.

Together our results led us to propose a model where module 12 acts as a sensory system without detoxification function (Summarized in Fig. 1 of Chapter 2). It controls a regulon which includes genes involved in controlling properties of the cell envelope (the *dlt* operon (D-alanylation of teichoic acids) and the *mprF* gene (L-lysynilation of phospholipids)) as well as the orphan ABC transporter encoding genes. Furthermore, ABC12 is as essential as TCS12 for

controlling the constitutive expression of these genes, but apparently not for a specific response to nisin. Finally, other regulatory systems besides module 12 control the expression of the *dlt* operon and *mprF* in *L. casei* BL23.

TCS have been characterized as regulatory mechanisms involved in acid resistance in lactic acid bacteria (Morel-Deville *et al.*, 1998; O'Connell-Motherway *et al.*, 2000; Azcarate-Peril *et al.*, 2005), albeit, the mechanisms by which they exert their effect remain to be determined in many cases. Modulation of the surface cell properties via D-alanylation of teichoic acids has also been described to play a major role in acid resistance in lactic acid bacteria as evidenced by the increased acid sensitivity observed in Dlt-defective mutants (Boyd *et al.*, 2000; Vélez *et al.*, 2007). Previous results had showed that strain ΔRR12 displayed a premature arrest of growth and it was very sensitive to low pH (Chapter 1). Subsequently, results reported in Chapter 2 showed that this defect was mainly due to low expression of the *dlt* operon and it could be alleviated by increasing the buffering capacity of the growth medium. These results suggested that TCS12 could be involved in the mechanisms that allow *L. casei* BL23 to adapt to acidic environments. On the basis of these results, the acid sensitivity of module 12 defective strains was addressed in order to gain insight into the effects of D-alanylation on the physiology of the cell envelope. TCS12 defective mutant was chosen because it displayed the same acid-sensitive phenotype than the Dlt-defective mutant but the latter displayed poor reproducibility in some assays. For that purpose in Chapter 3, “Characterization of the response to low pH of *Lactobacillus casei* ΔRR12, a mutant strain with low D-alanylation activity and sensitivity to low pH”, the ability to induce an acid tolerance response, fatty acid composition and proteome changes induced in the wild type and ΔRR12 strains in response to acid were investigated and compared.

Firstly, the ability to induce a growth phase dependent ATR of strains BL23 and ΔRR12 was determined. This growth phase dependent ATR is naturally induced as the pH of the media decreases as a consequence of the accumulation of acidic end-products of the metabolism (Buchanan and Edelson, 1996). The survival of *L. casei* BL23 and ΔRR12 after an acid challenge was determined. Results obtained showed that both strains elicited a growth

phase-dependent ATR at the end of the exponential growth phase, although the ATR induction was delayed in strain ΔRR12 compared to the wild-type strain BL23. Nevertheless, a similar trend was observed in the evolution of the growing culture pH in both strains and the same final pH was reached at the end of the assay. These data indicated that ΔRR12 impaired growth was not due to loss of metabolic activity and that inactivation of TCS12 did not impair ATR induction, however ΔRR12 is not able to increase the biomass of the culture and prematurely enters on stationary phase. The ATR in both strains was induced when the pH of the media decreased below 4.5. At this pH a substantial part of lactic acid is undissociated (23% in pure water) and can enter the cell by diffusion (Lambert and Stratford, 1999). Our results led us to suggest that ATR in *L. casei* is triggered when pH_{in} reaches a critical value as previously proposed for *Lc. lactis* (O'Sullivan and Condon, 1997). Additional experiments where designed to determine if the inactivation of TCS12 could be interfering cellular processes involved in the adaptation to grow at low pH of *L. casei*.

For this purpose, membrane FA composition was determined when the pH of the culture media reached 4.7, 4.3 and 4. In response to acid stress, bacteria change the membrane FA composition in order to decrease the proton permeability (Fozo *et al.*, 2004; Fozo and Quivey, 2004; Broadbent *et al.*, 2010). These changes usually involve an increase of the concentration of saturated FAs and CFAs. Our results showed that in both strains, the major FAs were palmitic, oleic, vaccenic and dihydrosterculic acids. The FA content varied with external pH in both strains: as the medium pH decreased, the content of unsaturated FAs decreased whereas the content of cyclic FAs increased in both strains. This indicates a tendency to decrease membrane proton permeability in both strains in response to media acidification. However, whereas the content of unsaturated FAs always was higher than that of saturated FAs in the wild-type strain, as pH decreased, the content of saturated FAs exceeded that of unsaturated FAs in the mutant strain ΔRR12. These results show that the FA content of strain ΔRR12 differs significantly of that of the parental strain BL23 and the changes in the content of some FA in response to decreasing medium pH also differed between strains. Thus, inactivation of TCS12 affected the membrane FA content.

In addition, differentially produced membrane associated proteins from cells exposed to pH 4.4 for 45 minutes were identified. This pH was chosen because ΔRR12 mutant prematurely enters in stationary phase around this value and significant differences in ATR induction between the wild-type and the mutant were observed when the pH of the media was between 4.5 and 4. The identification of differentially produced proteins revealed that they were putatively involved in a wide variety of cellular functions, thus indicating that the acid challenge induced global changes in *L. casei* BL23 physiology. The changes in the abundance of some proteins in both strains suggest that these proteins are involved in the response to acid of *L. casei* BL23. On the other hand, the abundance of some proteins varied when both strains were compared indicating changes brought about by the inactivation of TCS12.

Adaptation to low pH altered the abundance of proteins involved in peptide and amino acid metabolism as well as putative amino acid and peptide transporters both in the wild type and the mutant strain. Changes were also observed in both strains in the abundance of proteins participating in energy production. The increase of the α subunit and the dihydrolipoyl dehydrogenase of the pyruvate dehydrogenase (PDH) complex and the decrease of L-lactate dehydrogenase (Ldh) abundances after acid exposure led us to suggest that in *L.casei* BL23 there is an increase in the metabolic pathway from pyruvate to acetyl CoA by means of the PDH complex, and a reduction in the flux from pyruvate to L-lactate, in response to acid.

Although acid stress led to an increased abundance of two stress proteins in both strains, GroES and UspA, the trypsin-like serine protease HtrA was specifically induced in response to pH in the mutant strain ΔRR12. This result suggests that low pH damaged cell envelope proteins to a greater extent in strain ΔRR12 than in the parental strain.

An increased abundance of glyceraldehyde 3-phosphate dehydrogenase (Gap) was observed in strain ΔRR12 in response to acid. Interestingly, Antikainen et al. (Antikainen *et al.*, 2007) observed that Gap is associated to the cell envelope in *Lactobacillus crispatus* at acidic pH whereas at neutral pH is rapidly released to the media and that Gap interacts with negatively charged

LTAs. This led us to suggest that the increased abundance of Gap in strain ΔRR12 may therefore relate to low D-alanylation of LTAs in this strain compared to the parental strain BL23. Glutamine synthetase (*glnA*) was significantly more abundant in the mutant strain in response to acid exposure relative to reference conditions and it was also more abundant in the mutant relative to the wild-type in response to acid. Binding of GlnA to the cell envelope has also been described to preferentially occur at low pH (Kainulainen *et al.*, 2012) thus suggesting that GlnA also binds to LTAs. However, an increased association of Gap and GlnA with the membrane inside the cell could not be ruled out.

In summary, the results of Chapter 3 showed that although both *L. casei* BL23 and ΔRR12 induce a growth phase dependent ATR response, inactivation of TCS12 affects the ability of BL23 to adapt to acidic environments since the mutant prematurely enters into stationary phase whereas the wild-type strain keeps growing. Inactivation of TCS12 also led to significant differences in FAs and protein content of the cell envelope between both strains. In particular, the greater abundance of cytoplasmic proteins (such as Gap and GlnA) in the membrane fraction of the mutant strain ΔRR12 suggests an increased permeability of the cell membrane in this strain. All together our data suggest firstly, that D-alanylation of TAs has a remarkable influence on the functioning of the cell membrane of *L. casei,* and secondly that differences in acid tolerance between *L. casei* BL23 and its derivative strain ΔRR12 are possibly related to differences in surface physiology. Further experiments are required to elucidate this point and to integrate the information obtained so far.

The work presented in this PhD thesis must be understood within a global research context in the involvement of TCS in the stress response of the probiotic strain *L. casei*. Our results showed that TCS proved to be very versatile signaling mechanism in this microorganism. While TCS09 specifically senses and respond to AMPs, TCS12 plays a more general housekeeping role controlling genes involved in cell surface properties. Our results allow not only a better understanding of the signal transduction systems encompassing the stress response in *L. casei*, but also, a better understanding of the mechanism

that allow this bacteria to adapt and survive to changing environmental conditions.

FINAL CONCLUSIONS

1. TCS play a major role in the physiology of *Lactobacillus casei* BL23 and its adaptation to changing environmental conditions. TCS04 and TCS11 might regulate processes required for normal growth of *L. casei* BL23 whereas TCS01, TCS06 and TCS12 played a major role in stress response.

2. TCS09 and TCS12 are involved in AMP resistance in *L. casei* BL23. These systems constitute functional modules together with their cognate ABC transporters.

    2.1. Module 09 regulates the expression of ABC09 that possibly has a dual function of sensing and resistance to AMPs.
    2.2. Module 12 is an AMP sensory system where ABC12 does not have a direct role in AMP detoxification. The sensitivity of module 12 mutants to AMPs and their pleiotropic phenotype is caused by reduced expression of the RR12 regulated genes, in particular the *dlt* operon.

3. The ATR in *L. casei* BL23 is triggered when the intracelullar pH reaches a critical value corresponding to an extracelullar pH below 4.5.

4. In *L.casei* BL23 in response to acid there is an increase in the metabolic pathway from pyruvate to acetyl CoA by means of the PDH complex, and a reduction in the flux from pyruvate to L-lactate.

5. The inactivation of TCS12 and consequently the diminished expression of the *dlt* operon led to differences in the induction of the acid tolerance response, the cell membrane fatty acid composition and proteome changes during acid adaptation. This suggest that D-alanylation of TAs has a remarkable influence on the functioning of the cell membrane of *L. casei* and that differences in acid tolerance between *L. casei* BL23 and its derivative strain ΔRR12 are related to differences in surface physiology.

CONCLUSIONES FINALES

1. Los sistemas de dos componentes juegan un papel fundamental en la fisiología de *Lactobacillus casei* BL23 y en su capacidad de adaptación a condiciones ambientales adversas. Los sistemas TCS04 y TCS11 regulan procesos que afectan al crecimiento normal, mientras que los sistemas TCS01, TCS06 y TCS12 intervienen en la respuesta a estrés.

2. Los sistemas TCS09 y TCS12 participan en la respuesta a péptidos antimicrobianos (AMPs) en *L. casei* BL23. Estos sistemas constituyen módulos funcionales junto con los transportadores ABC asociados en el genoma.
   2.1. El módulo 09 regula la expresión del transportador ABC09 que posiblemente tiene una función doble en percepción y resistencia frente a AMPs.
   2.2. El módulo 12 es un sistema sensor de AMPs, donde el transportador ABC12 no participa directamente en detoxificación de éstos. La sensibilidad a AMPs de los mutantes en el módulo 12 y su fenotipo pleiotrópico se debe a una menor expresión de los genes regulados por el RR12, en particular el operón *dlt*.

3. En *L. casei* BL23 la respuesta adaptativa de tolerancia a ácido se induce cuando el pH intracelular alcanza un valor crítico que corresponde a un pH extracelular inferior a 4.5.

4. En *L. casei* BL23 en respuesta a ácido hay un aumento de las rutas metabólicas desde piruvato hacia acetil CoA, mediado por el complejo piruvato deshidrogenasa, y una reducción del flujo desde piruvato hacia L-lactato.

5. La inactivación del TCS12 y en consecuencia la menor expresión del operón *dlt*, produjo diferencias en la inducción de la respuesta de tolerancia a ácido, así como en la composición de los ácidos grasos de membrana y en el proteoma durante la adaptación a ácido. Esto sugiere que la D-alanilación de los ácidos teicoicos de la pared celular tiene un efecto importante en la funcionalidad de la membrana celular de *L. casei* BL23, y que las diferencias en tolerancia a ácido entre *L. casei* BL23 y la cepa mutante ΔRR12 están relacionadas con diferencias en el mantenimiento de la fisiología celular en estas dos cepas.

REFERENCES

Antikainen, J., V. Kuparinen*, et al.* (2007). "pH-dependent association of enolase and glyceraldehyde-3-phosphate dehydrogenase of *Lactobacillus crispatus* with the cell wall and lipoteichoic acids." J. Bacteriol. **189**(12): 4539-4543.

Azcarate-Peril, M. A., O. McAuliffe*, et al.* (2005). "Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in Lactobacillus acidophilus." Appl. Environ. Microbiol. **71**(10): 5794-5804.

Boyd, D. A., D. G. Cvitkovitch*, et al.* (2000). "Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity." J. Bacteriol. **182**(21): 6055-6065.

Broadbent, J. R., R. L. Larsen*, et al.* (2010). "Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress." J. Bacteriol. **192**(9): 2445-2458.

Bron, P. A. and M. Kleerebezem (2011). "Engineering lactic acid bacteria for increased industrial functionality." Bioeng Bugs **2**(2): 80-87.

Buchanan, R. L. and S. G. Edelson (1996). "Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells." Appl Environ Microbiol **62**(11): 4009-4013.

Collins, B., N. Curtis*, et al.* (2010). "The ABC transporter AnrAB contributes to the innate resistance of *Listeria monocytogenes* to nisin, bacitracin, and various beta-lactam antibiotics." Antimicrob. Agents Chemother. **54**(10): 4416-4423.

Coumes-Florens, S., C. Brochier-Armanet*, et al.* (2011). "A New Highly Conserved Antibiotic Sensing/Resistance Pathway in Firmicutes Involves an ABC Transporter Interplaying with a Signal Transduction System." PLoS ONE **6**(1): e15951.

De Angelis, M. and M. Gobbetti (2004). "Environmental stress responses in *Lactobacillus*: a review." PROTEOMICS **4**(1): 106-122.

Dintner, S., A. Staroń*, et al.* (2011). "Coevolution of ABC transporters and two-component regulatory systems as resistance modules against antimicrobial peptides in *Firmicutes* Bacteria." J. Bacteriol. **193**(15): 3851-3862.

Fozo, E. M., J. K. Kajfasz*, et al.* (2004). "Low pH-induced membrane fatty acid alterations in oral bacteria." FEMS Microbiol. Lett. **238**(2): 291-295.

Fozo, E. M. and R. G. Quivey, Jr. (2004). "Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments." Appl. Environ. Microbiol. **70**(2): 929-936.

Heimann, J. D. (2002). The extracytoplasmic function (ECF) sigma factors. Adv Microb Physiol, Academic Press. **Volume 46:** 47-110.

Hiron, A., M. Falord*, et al.* (2011). "Bacitracin and nisin resistance in *Staphylococcus aureus*: a novel pathway involving the BraS/BraR two-component system (SA2417/SA2418) and both the BraD/BraE and VraD/VraE ABC transporters." Mol. Microbiol. **81**(3): 602-622.

Jordan, S., M. I. Hutchings*, et al.* (2008). "Cell envelope stress response in Gram-positive bacteria." FEMS Microbiology Reviews **32**(1): 107-146.

Kainulainen, V., V. Loimaranta*, et al.* (2012). "Glutamine synthetase and glucose-6-phosphate isomerase are adhesive moonlighting proteins of *Lactobacillus crispatus* released by epithelial cathelicidin LL-37." J. Bacteriol. **194**(10): 2509-2519.

Lambert, R. J. and M. Stratford (1999). "Weak-acid preservatives: modelling microbial inhibition and response." J. Appl. Microbiol. **86**(1): 157-164.

Landete, J. M., L. García-Haro*, et al.* (2010). "Requirement of the *Lactobacillus casei* MaeKR two-component system for L-malic acid utilization via a malic enzyme pathway." Appl. Environ. Microbiol. **76**(1): 84-95.

Li, H., M. Lu*, et al.* (2010). "Protective Effect of Sucrose on the Membrane Properties of *Lactobacillus case*i Zhang Subjected to Freeze-Drying." Journal of Food Protection **73**(4): 715-719.

Mazé, A., G. Boël*, et al.* (2010). "Complete Genome Sequence of the Probiotic *Lactobacillus casei* Strain BL23." J Bacteriol **192**(10): 2647-2648.

Morel-Deville, F., F. Fauvel*, et al.* (1998). "Two-component signal-transducing systems involved in stress responses and vancomycin susceptibility in *Lactobacillus sakei*." Microbiology **144**(10): 2873-2883.

Neuhaus, F. C. (1985). "Inter-chain transacylation of D-alanine ester residues of lipoteichoic acid: a unique mechanism of membrane communication." Biochem Soc Trans **13**(6): 987-990.

O'Connell-Motherway, M., D. van Sinderen*, et al.* (2000). "Six putative two-component regulatory systems isolated from *Lactococcus lactis* subsp. *cremoris* MG1363." Microbiology **146 (Pt 4)**: 935-947.

O'Sullivan, E. and S. Condon (1997). "Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis*." Appl. Environ. Microbiol. **63**(11): 4210-4215.

Ohki, R., Giyanto*, et al.* (2003). "The BceRS two-component regulatory system induces expression of the bacitracin transporter, BceAB, in *Bacillus subtilis*." Mol. Microbiol. **49**(4): 1135-1144.

Ouyang, J., X. L. Tian*, et al.* (2010). "The BceABRS four-component system regulates the bacitracin-induced cell envelope stress response in *Streptococcus mutans*." Antimicrob Agents Chemother **54**(9): 3895-3906.

Palomino, M. M., M. C. Allievi*, et al.* (2013). "Osmotic stress adaptation in *Lactobacillus casei BL23* leads to structural changes in the cell wall polymer lipoteichoic acid." Microbiology **159**(Pt 11): 2416-2426.

Pfeiler, E. A., M. A. Azcarate-Peril*, et al.* (2007). "Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*." J Bacteriol **189**(13): 4624-4634.

Piuri, M., C. Sanchez-Rivas*, et al.* (2005). "Cell wall modifications during osmotic stress in *Lactobacillus casei*." J Appl Microbiol **98**(1): 84-95.

Rivas-Sendra, A., J. M. Landete*, et al.* (2011). "Response of *Lactobacillus casei* BL23 to phenolic compounds." J. Appl. Microbiol. **111**(6): 1473-1481.

Rowley, G., M. Spector*, et al.* (2006). "Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens." Nat Rev Microbiol **4**(5): 383-394.

Ruiz, N. and T. J. Silhavy (2005). "Sensing external stress: watchdogs of the *Escherichia coli* cell envelope." Curr Opin Microbiol **8**(2): 122-126.

Spano, G. and S. Massa (2006). "Environmental Stress Response in Wine Lactic Acid Bacteria: Beyond *Bacillus subtilis*." Critical Reviews in Microbiology **32**(2): 77-86.

Staroń, A., D. E. Finkeisen*, et al.* (2011). "Peptide antibiotic sensing and detoxification modules of *Bacillus subtilis*." Antimicrob. Agents Chemother. **55**(2): 515-525.

van de Guchte, M., P. Serror*, et al.* (2002). "Stress responses in lactic acid bacteria." Antonie Van Leeuwenhoek **82**(1-4): 187-216.

Vélez, M. P., T. L. A. Verhoeven*, et al.* (2007). "Functional analysis of D-alanylation of lipoteichoic acid in the probiotic strain *Lactobacillus rhamnosus* GG." Appl. Environ. Microbiol. **73**(11): 3595-3604.

Wu, C., J. Huang*, et al.* (2013). "Progress in engineering acid stress resistance of lactic acid bacteria." Appl Microbiol Biotechnol: 1-9.

Wu, C., J. Zhang*, et al.* (2012). "*Lactobacillus casei* combats acid stress by maintaining cell membrane functionality." J Ind Microbiol Biotechnol **39**(7): 1031-1039.