Cyclic di-nucleotide monophosphate cyclase in Firmicutes: from basic to practical approach

Dissertation

for the award of the degree

“Doctor rerum naturalium”

of the Georg-August-Universität Göttingen

Within the Binational Doctoral Program

on Molecular Biosciences and Biomedicine

Georg-August-Universität School of Science (GAUSS)

Universidad Nacional de Rosario (UNR)

Submitted by

Ingrid M. Quintana

From Rosario (Argentina)

Rosario-Göttingen, 2018

Thesis Committee

Dr. Christian Magni

(Instituto de Biología Molecular y Celular de Rosario, IBR CONICET)

Prof. Dr. Jörg Stülke

(Institute of Microbiology and Genetics, Department of General Microbiology)

Dr. Hugo Gramajo

(Instituto de Biología Molecular y Celular de Rosario, IBR CONICET)

Members of the Examination Board

Dr. Hugo Gramajo

(Instituto de Biología Molecular y Celular de Rosario, IBR CONICET)

Dr. Raúl Raya

(Centro de Referencia para Lactobacilos, CERELA CONICET)

Prof. Dr. Gerhard Braus

(Institute for Microbiology and Genetics, Georg-August-Universität)

Date of oral examination: June 11th, 2018

Herewith I declare that I have written the doctoral thesis entitled “Cyclic di-nucleotide monophosphate cyclase in Firmicutes: from basic to practical approach” on my own and with no other sources and aids than quoted.

Ingrid M. Quintana

Acknowledgements

I would like to thank in the first place my director Dr. Christian Magni and the whole LAB laboratory. It has been seven years of very hard work and extensive learning, surrounded by many people teaching me about science, partnership and teaching. Thanks Martin and Victor for your help, thanks to all the people I have met because of the lab. Very special thanks to Gabriela Martino, my science sister, who was my daily demonstration that teamwork and support is always possible.

I would also like to express my sincere gratitude to Facultad de Ciencias Bioquímicas y Farmacéuticas and the Universidad Nacional de Rosario. Free and lay tuition is something I will always be grateful for.

Natürlich möchte ich auch meinem Co-Direktor Prof. Dr. Jörg Stülke dafür danken, dass er mir die Möglichkeit gegeben hat, in einem so hervorragenden Labor zu arbeiten. Ebenfalls möchte ich PD Dr. Fabian Commichau und der gesamten Bacillus - Listeria - Mycoplasma Gruppe meinen Dank aussprechen; ein großartiger Motor, bei dem jede Verzahnung perfekt sitzt und arbeitet; diese gewonnenen Erfahrungen haben mein Leben verändert. Vielen Dank, Jan Gundlach, für die Hilfe bei meinen ersten Schritten in der c-di-AMP Welt und Christina Herzberg für Ihre Empfänglichkeit und Ihre experimentellen Ratschläge. Danken möchte ich außerdem Zuzanna Grubek, Jan Kampf, Cedric Blötz, Miriam Dormeyer, Johannes Gibhardt und Raphael Michna. Ich freue mich sehr, dass ich Sie getroffen habe und bin äußerst dankbar für Ihre Hilfe. Von Ihnen allen habe ich viel gelernt.

Special thanks to Nora Cascante-Estepa; no one has ever helped me so much, so selflessly. Silvia Castellon and Blanca Rincón, thank you too, for every conversation we had made my days brighter. Thanks to my Argentinian sister in Göttingen, Mariela Escobar, for every moment, trip, mate we shared.

I would also like to thank CONICET, ANPCYT, FBIOYF and DAAD for the financial support, as well as the Georg-August-Universität, and the staff from GAUSS, the CUAA-DAHZ and the Binational PhD program for all the assistance along the way.

Thanks to the magic of true friends, for putting me back on track in difficult moments and for sharing the happiness in good ones. Thanks to the University, El Poli, Folk and life itself for surruonding me with such wonderful human beings. I feel truly fortunate when I think I couldn’t mention you all because I would need another thesis. My very special thanks to

Acrolove and all the acroyoga Kula, evidence supporting I have family around the globe. Thank you for putting my head upside down, a smile in my face and siblings by my side: you restored my balance when the thesis was too heavy on my shoulders.

Gracias a mi familia, a mis padres y hermanos, cómo poner en palabras el idioma del corazón. Late por ustedes.

And last, but of course not least, gracias, thank you, vielen vielen Dank Chris, for your constant love, support and company, no matter what.

Table of contents

List of Publications

- **“c-di-AMP levels in *Lactococcus lactis*: overexpression of *cdaA* and *gdpP* can significantly modify this second messenger intracellular pool.”** Congress: Reunión conjunta de sociedades de biociencias. Noviembre, 2017. Buenos Aires, Argentina

- **“The KupA and KupB proteins of *Lactococcus lactis* IL1403 are c-di-AMP receptor proteins responsible for potassium uptake.”** Quintana I., Grubek Z., Gibhardt J., Lee V. T., Commichau F. M., Stülke J., and Magni C. (2018) Applied and Environmental Microbiology. Sent.

- **“Genetic engineering of *Lactococcus lactis* co-producing antigen and the mucosal adjuvant 3´ 5´- cyclic di adenosine monophosphate (c-di-AMP) to design a mucosal vaccine prototype.”** Quintana I., Espariz M., Stülke J., Villar S., González F., Pacini F., Cabrera G., Bontempi I., Procheto E., Perez A. R., Marcipar I., Blancato V., and Magni C. (2018) Frontiers in Microbiology. Sent.

Summary

Cyclic di-adenosine monophosphate (c-di-AMP) is a second messenger involved in diverse metabolic processes such as cell wall homeostasis, biofilm formation, antibiotics and heat resistance, among others. In *Lactococcus lactis* and *Enterococcus faecalis*, Lactic Acid Bacteria used not only as research models but also as a cell factory in biotechnological processes, the only reported interaction partner of c-di-AMP is the pyruvate carboxylase enzyme, PyrCarb. Nevertheless, in the last year investigations directed its main role towards potassium metabolism.

In this thesis, KupA and KupB, two potassium transporters encoded in *L. lactis* IL1403 genome, are described for the first time. According to an *in silico* analysis, these proteins, which belong to the Kup/HAK/KT family, are highly conserved in this species, being therefore a strain independent potassium uptake system. In addition, evidence shows that both proteins are able to uptake this cation with high affinity, and we demonstrate that KupA as well as KupB bind to and are down-regulated by c-di-AMP.

On the other hand, different strains derived from *L. lactis* IL1403 were developed aiming to modify intracellular pools of c-di-AMP in a stable system. One strategy for the reduction of c-di-AMP levels was the obtaining of *ΔgdpP* mutants via homologous recombination. Maintenance of this second messenger levels close to wild type ones, suggested the presence of another c-di-AMP degrading enzyme. A first description of a putative enzyme with this activity, encoded by *yheB* gene was done by BNPP assay. In addition, by use of a pH inducible vector, construction of strain *L. lactis* LL03 with concentrations of this second messenger above 15 times basal levels was possible. This system was therefore selected for further investigations on the development of a vaccine prototype against Chagas disease.

On the other hand, *L. lactis* is a promising candidate for the development of mucosal vaccines with more than 20 years of experimental research. Moreover, c-di-AMP has been reported as a strong mucosal adjuvant promoting both humoral and cellular immune responses. Altogether, in this thesis the development of a recombinant *L. lactis* strain is reported, able to produce both an antigen as well as an adjuvant in order to develop a novel vaccine prototype against the *Trypanosoma cruzi* parasite, the causal agent of Chagas disease. This is a tropical disease originated in a specific area of South America but currently spreading in four continents.

Finally, an initial approach was done on c-di-AMP metabolism in *E. faecalis*. The presence of a Kup transporter was also corroborated in this species, and some basic characteristics of the c-di-AMP degradative pathway were explored via a *ΔgdpP* mutant construction. Finally, the impact of GdpP on the virulence of *E. faecalis* was analyzed by use of the infection model *Galleria mellonella*.

1. Introduction

"Remember to look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist" Stephen Hawking

1.1. Lactic Acid Bacteria

Along the course of history, human beings always sought for efficient ways of improving quality of everyday life. When food supplies were not round the corner, one of these ways concerned food preservation. In this sense, a wide variety of products including meat, cheese, vegetables and dairies to name a few, were well conserved thanks to the process of fermentation. Successful results were not investigated, there was a lot of variation from one fermented product to the next one and optimization of these procedures was obtained after trial and error. A widely practiced technique consisted on separating a small portion of a successful batch of a fermented product and using it to start the process in fresh food. This “back-slopping” method allowed people to maintain food in good conditions, by transferring the microorganisms which made fermentation possible. In lactic fermentation, the most represented amount of bacteria conforming the inocula were Lactic Acid Bacteria (LAB) (1) .

Nowadays, mechanisms of preservation are thoroughly understood and different combinations of LAB are used to inoculate a culture, expecting specific features in the final fermented product. In the cheese making industry, for instance, certain strains are used as starter or adjunct cultures to contribute to flavor and aroma development, or to other specific organoleptic features such as the formation of eyes via CO_2 release (2) . Also, proteolysis and lipolysis are key pathways for texture and flavor improvement and so is the formation of C4 aroma compounds like acetoin and diacetyl, both derivatives of lactic acid (3).

The group of LAB cannot be addressed as microorganisms falling in one simple definition. Instead, they are referred to as a group of bacteria with certain key characteristics in common. Principally, the high amounts of lactic acid they produce when grown in the presence of a suitable carbon source, as result of homofermentation or heterofermentation, in which case they produce acetic acid and ethanol as well. These acidic compounds and the concomitant decrease in pH are one of the main reasons for the growth inhibition of undesired microorganisms. Moreover, LAB have less than 50% of G+C content in their genomes and they are non-sporulating Gram-positive bacteria. They are strictly fermentative, anaerobic or aerotolerant and morphologically they can be cocci or rod shape bacteria. Phylogenetically, LAB genera are all included in the firmicutes phylum, except for the *Bifidobacterium* genus, belonging to *Actinobacteria.* Genera within *Firmicutes* comprise: *Aerococcus*, *Aloiococcus*,

Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and *Weisella*, among others (4).

Due to its historical utilization in food industry, they have been classified as "Generally Regarded as Safe" (GRAS) by the Food and Drug Administration of the United States of America (FDA) and they have also been designated with the "Qualified Presumption of Safety" (QPS) according to the European Food Safety Authority (EFSA). Consequently, in the last decades increasing interest and lines of investigations have been opened concerning other fields and applications of LAB, which is how from food preservation and starter cultures they expanded into probiotics development, enzymes production, drugs delivery, vaccines formulation, among others.

Nevertheless, within the LAB group there are also some controversial species, which are pathogens or opportunistic pathogens, like members of the *Streptococcus* and *Enterococcus* group (Fig. 1). Even though some strains of *Enterococcus* are able to produce bactericidal compounds and therefore their biotechnological applications are encouraged, special care need to be taken when working with microorganisms prone to acquire antibiotic resistances and turn into nosocomial pathogens (see below).

Members of LAB, apart from the acidifying effect on growth media as previously mentioned, are also efficient inhibitors of undesired microorganisms due to the synthesis of bacteriocins. These are peptidic compounds with bactericidal effects on closely related bacteria from the same ecological niche, which provides an evolutionary advantage to the producer strain (5). Its effects have also been proven in more distant species, mainly when environmental conditions can be adjusted to favor the bacteriocin mode of action. The first recognized bacteriocin synthesized by *L. lactis* was nisin, which has been employed for decades in food industry for its antimicrobial action against members of Gram-positive bacteria. Noteworthy, it also inhibits growth of *Clostridium* and *Bacillus* spores, and different combinations of nisins, nisin producer starter strains and other preservation mechanisms have been applied in food industry and fermentation processes without health risks for consumers (6, 7).

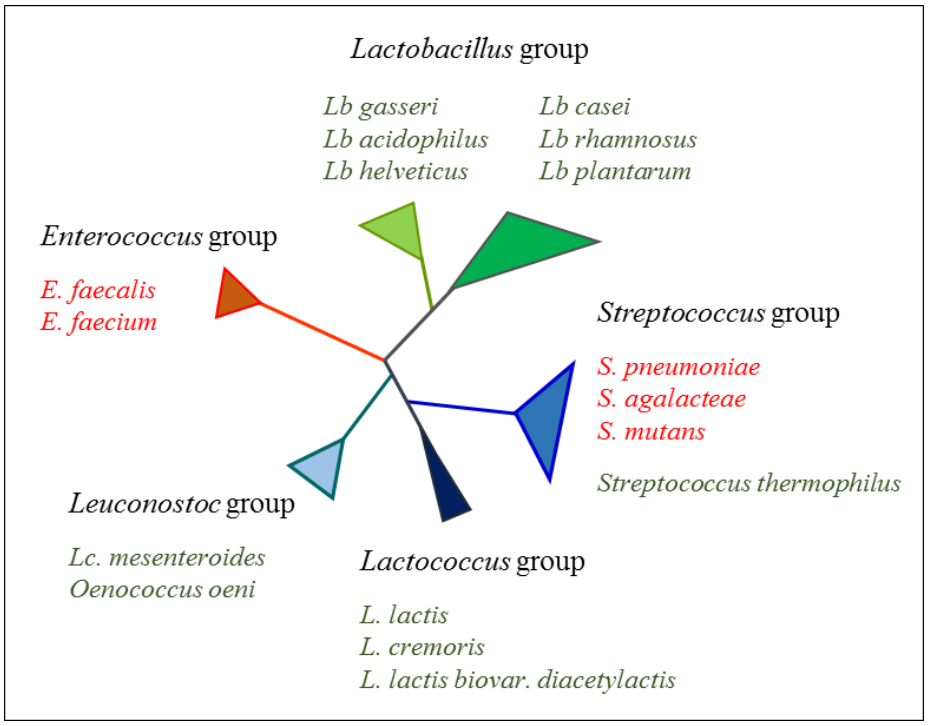

Fig. 1 Schematic phylogenetic tree of LAB based on 16 rRNA gene sequence homology. Representative LAB species related to the food industry are included. Species that do not represent a risk for human health are depicted in green, opportunistic pathogens in red.

Other bacteriocins active against pathogenic bacteria in human infections have prompt the development of probiotics. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (8). The intestinal lumen is an elaborated matrix with complex microbiota that varies for each host and is influenced by different direct and indirect agents. Examples of indirect agents are the use of antibiotics and also the diet, which has been referred to as the most efficient way of modulating gut microbiota (9) . Diets complemented with probiotics and symbiotics have a beneficial impact on the immune system of individuals with infectious bacterial diseases and even a connection was established between the increase of *Lactobacillus* levels and the vitamin A supplementation for Norovirus treatment (10). Interesting results have been obtained as well for *Helicobacter pylori* inhibition by use of LAB isolated from fermented noodle (11), and bacteriocins synthesized by LAB have also been reported to be active against pathogenic bacteria like *Listeria monocytogenes*, *Staphylococcus aureus* and *Streptococcus mutans* (12, 13).

1.1.1. *Lactococcus lactis*

Lactoccus lactis is one of the most used starter culture in the cheese making industry as well as the best characterized LAB (14). Several strains have been thoroughly studied for their impact on the fermented final product, and they fall mainly into two subspecies, *L. lactis* subsp. *cremoris* (preferentially used as starter since they cause less bitterness), and *L. lactis* subsp. *lactis* (known as a fast acidifier). A biovariety can also be found within *L. lactis* species, i. e. *L. lactis* subsp. *lactis* biovar. *diacetylactis*, capable of citrate fermentation and therefore affecting flavor development via acetoin and diacetyl synthesis (15).

From an evolutionary point of view, *L. lactis* has been able to colonize different ecological niche, like plant surfaces, the urogenital and gastrointestinal tract of mammals as well as other animal tissue surfaces. It can also be found in different types of foods including dairies, meats and fermented products. Strains from diverse sources have evolve loosing or gaining specific sets of genes which made possible their adaptation to the defined media employed in industries, the rich media of dairy matrices and the most demanding natural environments of non-dairy sources such as plants. Consistent with this, a recent study performed on 30 sequenced genomes available on the NCBI database belonging to *L. lactis* taxon, reported that 16% of the unique gene families of each genome correspond to phage proteins acquired through the integration of a particular pro-phage element (16). Moreover, about 19% of these gene families showed to be involved in mobilization and conjugation, transposases and IS elements, or genes encoding systems providing a beneficial niche specific advantage related to bacteriocin production, sugar metabolism and restriction-modification systems. This study also showed a clear division between *lactis* and *cremoris* subspecies, and allowed the determination of the core genome sizes being of 1406 and 1413 genes, respectively (plasmids were excluded from the study) (16). For industrially relevant *L. lactis* strains, the most important set of genes are those coding for proteins that enable lactose fermentation, the prevalent carbon source in dairy products. These genes are encoded in the *lacABCDEFGX* operon, which is found in plasmids of all *cremoris* subspecies, except for the plasmid free strain MG1363 and its derivative NZ9000. On the other hand, within *lactis* subspecies the non-degrading lactose strains are more represented, being IL1403 one of these. Altogether, *L. lactis* is a very versatile species with a wide research history and range of options for industrial applications, which are currently under investigation.

L. lactis subsp. *lactis* IL1403 (from now on *L. lactis* IL1403) was the first sequenced *lactis* strain, and it is the plasmid free version of strain IL594 isolated from a cheese starter culture (17). Together with strain NZ9000 and MG1363, it is the most used genetic and physiological *L. lactis* model in the laboratory (14) (for a summary of *L. lactis* strains used for research and industrial applications see Fig. 2 and Table I). *L. lactis* IL1403 has been employed in research for decades and its consequent adaptation and domestication led to a small genome of approximately 2500 open reading frames (ORF), nearly the half of other well-known bacterial models such as *Bacillus subtilis*. As a member of LAB, this strain is expected to be strictly fermentative, but genes related to aerobic respiration have been found in *L. lactis* IL1403 genome (18). Namely, *men* and *cytABCD* operons are present, involved in menaquinone synthesis and cytochrome d biogenesis respectively, and even *hemH*, *hemK* and *hemN* for late steps of heme biosynthesis (oxidation of porphyrinogen and attachment of iron to heme), although no genes for the early steps were identified.

L. lactis ssp. *cremoris* MG1363 was initially obtained as the plasmid cured progeny of the dairy origin NCD0712 strain (19) and it is today the model laboratory strain for genetic engineering and biotechnology of *L. lactis* (Fig. 2). From this strain also derives one fundamental tool for the nisin-controlled gene expression system (NICE), which was obtained via replacement of *pepN* gene (coding for an aminopeptidase) for the *nisK* and *nisR* genes (20), encoding a histidine-kinase and a cytoplasmic response regulator, respectively. The strain thus created, named NZ9000, is able to sense nisin in the medium upon which NisK autophosphorylates and transfers the phosphate to the counterpart of the two-component system, NisR, being thus activated. The active version of NisR induces in turn the P*nisA* promoter, present in the pNICE series of vectors, for which expression of a gene of interest cloned under it is successfully induced (21). It is important to mention that *L. lactis* NZ9000 lacks 1821 nucleotides in the *pepN-napC* locus and when compared to the original strain MG1363, it has 6 point mutations independently acquired, being otherwise genetically equivalent (22).

Another tool developed for *L. lactis* engineering and biotechnology, of special relevance for this work, is strain *clpP-htrA*, which shows reduced proteolytic activity, due to the lack of both major proteases Clp and HtrA (Fig. 2). Consequently it is a good strategy for the stable production of a heterologous protein of interest (23).

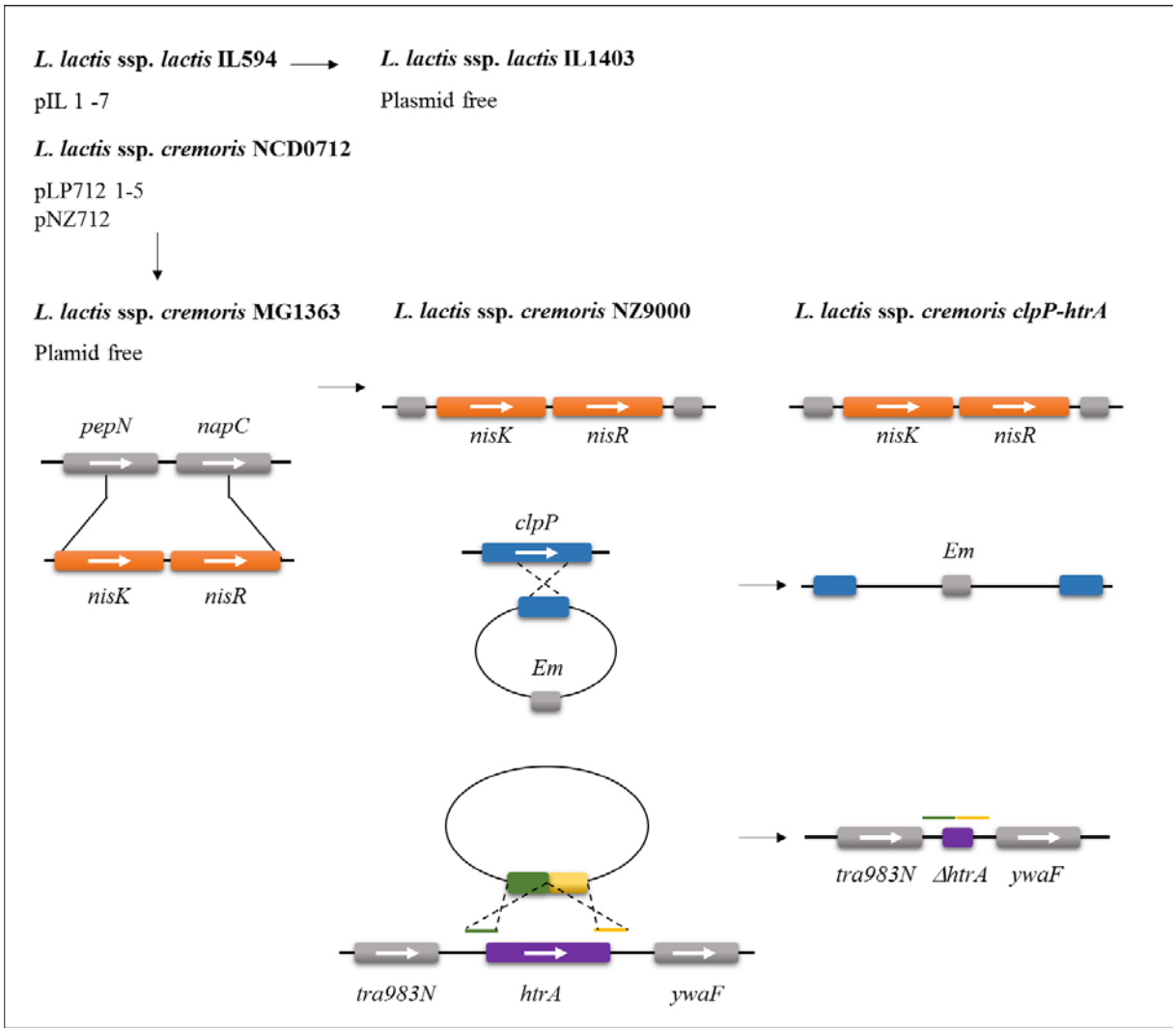

Fig. 2 Representative diagram of the development of *L. lactis* strains commonly used for genetic engineering and gene expression. *L. lactis* IL594 contains seven plasmids (pIL1 to 7), and is the parental strain of the plasmid free IL1403 strain. On the other hand, *L. lactis* MG1363 is the free plasmid version of strain NCDO712. It is also the parental strain of NZ9000, which carries the *nisKR* insertion, for nisin sensing and activation of the NICE system vector series. Strain *clpP-htrA* lacks the proteolytic enzymes HtrA and ClpP due to a deletion in the former case, and an erythromycin gene replacement, in the latter.

1.1.2. *Enterococcus faecalis*

Enterococcus faecalis is a natural commensal member of the human gut flora, and a very versatile bacterium for diverse niches colonization. As a member of the *Enterococcus* genus, it can grow in temperatures ranging from 10º C to 45º C, it can survive acidic and alkaline conditions (from pH 4.0 to 9.6), and can resist high salt concentrations (up to 6.5% NaCl) (24). *E. faecalis* can be found in plant materials and foods, especially in fermented products of animal origin, although in water and non-fermented meat articles its presence is not desired and normally it is used as an indicator of sanitary quality (25).

E. faecalis is as well one of the most controversial LAB, since it can participate in DNA exchange, being able to acquire antibiotic resistances, genes related to haemolysin-cytolysin production, proteins involved in adhesion to host tissue, or other virulence factors (26). The most complicated strains are the vancomycin resistant, which normally enter the host via wounds and catheters, causing endocarditis, bacteraemia and urinary tract infections (27). Nevertheless, due to *E. faecalis* wide genetic variability, strains isolated from clinical samples are significantly different from those isolated from food. Abriouel et al. showed that species most frequently involved in human diseases are also most frequently associated with clinical samples and in the same study enterococcal isolates from fruits and vegetables showed a much lower incidence of antibiotic resistance compared to clinical samples (28).

Notwithstanding its controversial profile, it is a reality that *Enterococcus* (mainly *E. faecalis*, *E. faecium* and *E. durans*) are part of food products, either due to contamination along the manufacturing process or as part of dairy starter, adjunct and/or non-starter cultures (29). Moreover, *E. faecalis* is the species with the most acidifying, proteolytic and lipolytic activities, which as previously mentioned, exerts a strong contribution to flavor development. It is well known nowadays that its presence in food products like cheese and fermented meats contributes to the ripening process in the development of favorable organoleptic features (30).

Some strains of *E. faecalis* have even been reported as probiotics and others are known for its bacteriocin production (31). *E. faecalis* synthesizes enterocin AS-48, which has a broad inhibitory spectrum, and offers interesting possibilities for food preservation. It has been shown to be active against Gram-positive bacteria like *L. monocytogenes*, *S. aureus*, *Mycobacterium*, *Bacillus cereus* and even some Gram-negative bacteria (32-34).

1.2. Potassium homeostasis

Ion homeostasis is a key factor for life in the three kingdoms. Particularly, potassium (K^+) is the most abundant cation in the cytosol, and its uptake is essential and tightly regulated in all living cells. It intervenes in important cellular processes like translation, being essential for prokaryotic 50S and 30S ribosomal subunits activity, in charge of the peptidyl transferase reaction and the binding of phenylalanine-transfer RNA *in vitro*, respectively (35, 36). Potassium concentration is a limiting factor for protein synthesis, and it has been reported that the rate of protein synthesis slows down fourfold when K^+ concentrations are under 25 mM (37).

Many enzymes require potassium as cofactor and it is also linked to bacterial bioenergetics, since it affects both components of the proton motive force (PMF), the pH gradient (ΔpH, alkaline inside the cell) and the electric potential across the cellular membrane (Δψ, negative inside the cell) (38). Secondary transporters involved in ΔpH generation, generally use the PMF generated by respiration or ATPases to actively uptake protons in exchange for cytoplasmic cations like Na^+ or K^+ (39). Therefore, potassium metabolism also affects pH homeostasis via cation–proton antiporters, especially crucial for non-respiring LAB, which acidify growth media and need to maintain alkaline values of pH inside the cells when extracellular values can go down to lower limits than their growing capacity (40).

The Δψ component of the PMF is also related to potassium metabolism since in alkaline pH homeostasis, it enables the electrogenic influx of protons in exchange of an unequal ratio of Na^+ or K^+ efflux. Nevertheless, Na^+ /H^+ antiporters are more often involved in this process, whereas K^+ /H^+ antiporters take relevance under Na^+ limitation (39, 41). In turn, this membrane potential is also important for ATP production and nutrient uptake, for which potassium metabolism influences cell growth as well.

Cells also need a mechanism to alter their intracellular K^+ concentrations in response to external solute changes. In these respect, potassium channels play a crucial role in osmotic stress responses. Most bacteria growing at low osmolarity use K^+ as primary osmotic solute and when grown in high osmolarity media, potassium acts as a second messenger to trigger accumulation of compatible solutes. (42). In this sense bacterial cell turgor and cell viability under variable environmental conditions are maintained broadening colonization possibilities.

It is important to mention that osmoregulation is also affected by the pool of compatible solutes. These molecules help reaching homeostasis after osmotic changes in the environment. Water efflux when osmolality increases outside the cell is first coped with potassium intake. Nevertheless, this is unsustainable when prolonged in time since it can disturb protein synthesis and other cellular processes and interactions affected by K^+. Bacteria have therefore evolved developing a second phase of adaptation to osmotic stress, by synthesis of osmoprotectant solutes like betains, polyols, sugars, amino acids and compounds derived from them (43). A complex matrix is therefore established around potassium metabolism, connecting different cellular pathways in order to maintain homeostasis.

1.2.1. Potassium uptake systems

Bacteria have evolved different systems to accumulate potassium intracellularly and achieve stable concentrations in order to cope with basic metabolic demands (40). Different high and low affinity uptake mechanisms are involved, if the bacteria are growing in media with low or high K^+ concentrations, respectively.

In *E. coli*, intracellular concentrations of K^+ are kept close to 200 mM, when concentration in growth media is 10 mM (31). In this bacterium, there are three main potassium transport systems: Trk, Kdp, and Kup. Together they ensure potassium uptake in different growth conditions, and although mutants in the three systems cannot grow in regular media (unless at least 115 mM K^+ is added), one of them is already enough for growth (44, 45).

Trk system consists of four genes, which are constitutively expressed, with *trkA* as the main component and its product the predominant K^+ transporter at neutral pH. It has low affinity for the ion and depends on ATP and proton motive force for potassium uptake (44). On the other hand, the KdpATPase system includes the inducible high affinity transporter KdpA, which is synthesized at low K^+ concentrations and under osmotic stress (46). Finally, Kup (formerly TrkD) is also a constitutive but low affinity system and its activity increases at low pH, when TrkA and Kdp activities are insufficient (47).

In other model bacterium like *B. subtilis*, *S. aureus* and *L. monocytogenes*, one of the main systems in charge of potassium uptake is Ktr, which like Trk belong to the Trk/Ktr/HKT family (48). Moreover, two different affinity systems can be distinguished within constitutive Ktr transporters: KtrCD is a low affinity system present in the three bacteria mentioned, whereas high affinity KtrAB is also present in *B. subtilis*, where it is regulated by the *kimA* riboswitch. Interestingly, another copy of the riboswitch can be found in *B. subtilis*, regulating as well *kimA* (formerly *ydaO*), coding for a member of a recently discovered potassium transporter family (49). An increase of K^+ concentration results in accumulation of c-di-AMP which binds to the riboswitch and consequently represses expression of the downstream encoded transporters (49). Interestingly, the second messenger c-di-AMP (described in the following section) was proved to bind subunits KtrA and KtrC as well, upon which inhibition of the respective transporters occurs (50).

As previously mentioned, potassium metabolism is an intricate network affecting diverse pathways, and so is potassium uptake. Even though a brief outline is made here as

regards major K^+ transporters in model bacteria, it cannot be ignored that other minor systems exist as well.

1.3. (3',5') cyclic-di-adenosine-monophosphate

(3',5') cyclic-di-adenosine-monophosphate (c-di-AMP) is a second messenger recently discovered in the crystallographic structure of DisA, a protein in charge of scanning DNA integrity in *Thermotoga maritima* (51). Therefore, the first function assigned to this compound was related to the DNA-damage dependent cell cycle control. Later it was also linked to cellular growth and cell wall homeostasis (52) as well as sporulation (53) and other cellular pathways like antibiotic resistance and nitrogen metabolism, among others (54).

In pathogenic organisms, it was reported that c-di-AMP has an important role in the establishment of infection. For instance, in *L. monocytogenes*, c-di-AMP secretion after infection induces the immune response mediated by interferon β (55) and in *Streptococcus pneumoniae* it was observed that a double mutant strain lacking c-di-AMP degrading enzymes GdpP and PgpH (described below) is no longer capable of infection. Moreover, both proteins are involved in four key stages of pneumococcal pathogenesis: colonization, otitis media, pneumonia and bacteremia (56).

c-di-AMP homeostasis is tightly controlled; low concentrations as well as its accumulation are detrimental for the cell. In *B. subtilis*, high levels of c-di-AMP lead to mutations that inactivate the activity or expression of its synthesizing enzyme, CdaA (see below) (54), and in *L. monocytogenes*, a double mutant in GdpP and PgpH results in growth defects inside and outside the host (57). Moreover, *S. aureus* strains lacking GdpP increase intracellular concentrations of c-di-AMP and are more resistant to acid stress and in general, higher concentrations of this second messenger are associated to increased resistance to β-lactam antibiotics (52, 58, 59).

The main interest on this compound was related to its essential role in rich media for low GC Gram-positive bacteria, since only in 2015 it was possible to obtain a mutant strain for the only c-di-AMP synthesizing enzyme in *L. monocytogenes*, in strictly controlled minimal media (60).

In the last years, investigations were centered mainly in the role of c-di-AMP in potassium homeostasis and osmolyte transport, and even though its interaction to potassium

transport components was previously proven (50, 61), it was only recently that a direct connection was made between K^+ metabolism and c-di-AMP essential role (49). In *B. subtilis*, it was shown that this second messenger also regulates synthesis of KimA (K^+ importer A) via the *kimA* riboswitch, a copy of which is also found upstream *ktrA*, regulating its expression as well. Moreover, in the same study, a *B. subtilis* strain lacking c-di-AMP synthesizing enzymes was obtained under extremely low potassium concentrations (49) and in *S. aureus* this was possible in chemically defined medium and in rich medium supplemented with sodium or potassium chloride (62).

1.3.1. Synthesis of c-di-AMP

c-di-AMP is synthesized by specific di-adenilate cyclases from two ATP molecules, with the exception of *M. tuberculosis*, which can also produce it from two molecules of ADP (63). Three types of DAC containing enzymes are so far described, and they are all present in the model organism *B. subtilis*. The cyclase enzyme DisA, can be found in actinobacteria and in spore-forming firmicutes, and it associates into two interacting tetramers. It is involved in DNA integrity control and repair, being inhibited by damaged DNA as well as atypical DNA arrangements such as Holliday junctions (51). On the other hand, CdaS cyclase is only found in spore forming *Bacillus* members and in one *Clostridium* species, and it is exclusively expressed during sporulation, guaranteeing spores efficient germination. It contains two α-helices at the N-terminal end, followed by the DAC domain at the C-terminus (64) The third c-di-AMP synthesizing enzyme, CdaA, is the most frequently found in firmicutes. Similarly to CdaS, the DAC domain is located at the C-terminal end, whereas three α-helices can be found at the N-terminus (Fig. 3). It is important to mention that DAC containing proteins are extensively distributed in both Gram + and – bacteria, being much frequently found in the latter, and normally associated to additional domains in charge of the input and output of a variety of signals. Nevertheless, in the vast majority of low GC firmicutes, only one protein with a DAC domain is typically found.

In many δ-proteobacteria and firmicutes, *cdaA* is encoded in a widely conserved gene cluster (described for *L. lactis* in section 3.1.6., Fig 42), which comprises *cdaR*, codifying for a CdaA regulatory protein, and *glmM*, coding for the phosphoglucosamine-6-phosphate mutase enzyme, capable of converting α-D-glucosamine-6-phosphate into D-glucosamine-1-phosphate, a cell wall precursor. It is therefore not surprising that one of the first pathways

associated to c-di-AMP was cell wall biosynthesis, since genes responsible for the synthesis of related proteins are normally clustered together in the DNA.

As previously mentioned, c-di-AMP is essential when bacteria are grown in rich media. Consequently, many efforts are being directed into the development of drugs able to inhibit c-di-AMP cyclase activity and being therefore suitable for antibiotic formulation. For example, the anti-parasitic urea-derived drug suramin (used for African trypanosomiasis and onchocerciasis treatment) is proven to inhibit *T. maritima* DisA, competing with ATP for the same binding site in the enzyme (65).

Altogether, c-di-AMP synthesis and metabolism are involved in diverse cellular pathways, and even though an increasing amount of information as regards this second messenger is being generated nowadays, its potential applications continue to expand.

1.3.2. Degradation of c-di-AMP

Bacteria capable of c-di-AMP synthesis also contain specific hydrolyzing enzymes in charge of its degradation. These proteins are phosphodiesterases, and can be divided into two families. The first family comprises homologues of a membrane-bound protein named GdpP, which consists of two transmembrane helices, a degenerate PAS domain (Per-Arnt-Sim, for signal transduction, (66), a modified GGDEF domain and a DHH-DHHA1 domain with a catalytic motif Asp-His-His. On the other hand, the second family includes an HD domain, where a His-Asp motif can be found, and it is separated of an N-terminal extracellular domain by seven transmembrane helices (Fig. 3) (67). Homologues of this group are distributed in cyanobacteria, bacteroidetes, fusobacteria, species of the genus *Thermotoga* and members of firmicutes, where proteins of both families are normally found together.

The most studied phosphodiesterase, GdpP, is present in firmicutes, actinobacteria and spirochaetes. It degrades c-di-AMP into the dinucleotide 5’pApA and belongs to the DHH-DHHA1 family along with another kind of phosphodiesterase, found for instance in *S. pneumoniae*, presenting a unique soluble DHH/DHHA1 domain (68). This enzyme is capable of degrading 5’pApA into two molecules of AMP and it is a *B. subtilis* NrnA homologue, although in this microorganism it does not hydrolyses c-di-AMP. This single domain version is of course smaller and cytoplasmic, and it is present in *Borrelia burgdorferi* y *M. tuberculosis* as the only c-di-AMP degrading phosphodiesterase (69, 70).

Phosphodiesterases from both groups have been proved to be controlled by the signaling nucleotide guanosine-tetraphosphate (ppGpp), establishing a link between c-di-AMP metabolism and the stringent response, designed to conserve energy during nutrient starvation. Both GdpP and PgpH (HD family) have been proved to be strongly inhibited by ppGpp (71, 72)

Biochemical characterizations have shown that phosphodiesterases have multiple functions and regulatory inputs, including ppGpp, heme group and nitric oxide union and ATPase activity (73). Moreover, *gdpP* mutants of *S. aureus* and *B. subtilis* were significantly more resistant to β-lactamic antibiotics and in general, for mutations in this enzyme phenotypes of stress resistance to pH, high temperatures and compounds affecting cell wall stability were obtained (58, 74, 75).

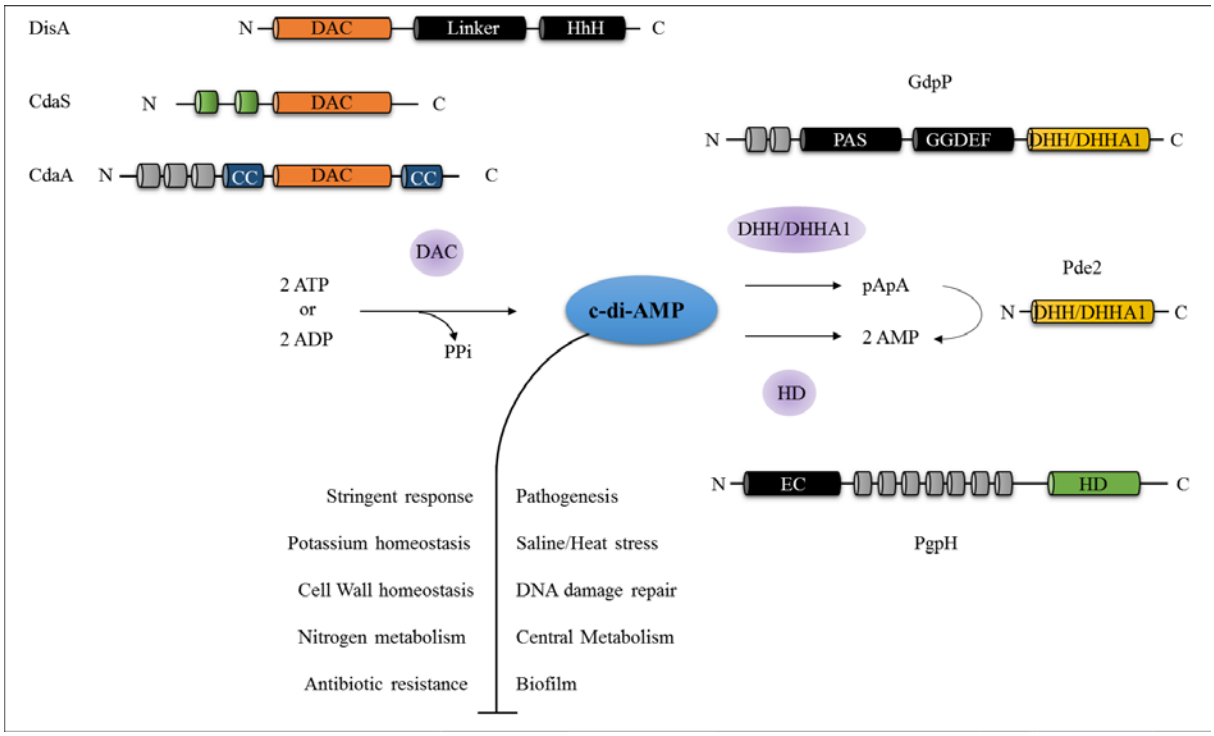

Fig. 3. Representation of c-di-AMP synthesis and degradation. DAC containing enzymes (orange) are in charge of c-di-AMP synthesis. The three types of cyclases are represented: DisA, CdaA and CdaS. c-di-AMP is synthesized from two molecules of ATP, although *M. tuberculosis* and *S. mutans* can also use two molecules of ADP as substrate. The two phosphodiesterases families are depicted on the right: HD (green), hydrolyzing c-di-AMP to AMP and DHH/DHHA1 (yellow). Within this family two types of enzymes can be distinguished: the most frequently membrane-bound version, which yields pApA, and the smaller soluble version, which can further degrade c-di-AMP to AMP. Principal pathways related to c-di-AMP metabolism are also indicated.

1.3.3. c-di-AMP interaction partners

The first reported c-di-AMP interaction protein was DarR of *Mycobacterium smegmatis.* This protein is a transcriptional factor of the TetR family, which is stimulated by c-di-AMP and consequently binds to the promoter region of its own gene, repressing its expression. It also represses expression of a divergent operon, involved in fatty acid metabolism and of *cspA* gene, encoding a cold shock protein (76).

As previously described, this second messenger has a strong influence in potassium homeostasis and its interaction to different potassium transporters has been reported. c-di-AMP binds to KtrA, the cytoplasmic regulatory component of the KtrAB potassium uptake system. The binding site is the regulator of conductance of K^+ (RCK_C) domain, oriented towards the cytosol, and upon binding, it inhibits potassium uptake in *B. subtilis*, *S. aureus* and *S. pneumoniae* (50, 77). Interestingly, c-di-AMP in *B. subtilis* also binds to the *kimA* riboswitch, which regulates expression of *ktrA* as well as *kimA*, coding for a recently reported potassium importer (49). Moreover, in *S. aureus* this second messenger regulates all potassium uptake systems so far described, which includes KtrC and the KdpD subunit of the KdpDE two component system previously described (50, 61).

Noteworthy, the RCK domain is present in a wide variety of proteins, like CpaA of *S. aureus*, a putative cation/proton antiporter recently proven to be regulated by c-di-AMP as well. Contrary to the c-di-AMP binding proteins so far described, this second messenger activates CpaA in *S. aureus* (78). On the other hand, PstA, a PII like signal transduction protein, present in *S. aureus*, *L. monocytogenes* and *B. subtilis* (where it is named DarA) also binds c-di-AMP, and even though several investigations were carried to elucidate its interaction to c-di-AMP, its function remains unknown (50, 54, 79).

As stated, osmoregulation involves not only potassium homeostasis but also regulation of the synthesis and uptake of other osmolytes. c-di-AMP has been related to osmolarity homeostasis in both ways, i.e. potassium intake and osmoprotectants metabolism. For instance, c-di-AMP also interacts with OpuCA, the ATPase component of the carnitine ATP-binding cassette transporter OpuC (80). Interestingly, binding of c-di-AMP to OpuC occurs in the cystathionine-β-synthase (CBS) domain, broadening the possibilities of interactions to CBS-containing, like *L. monocytogenes* CbpA and CbpB proteins of unknown function (81).

In *L. monocytogenes*, c-di-AMP also binds to CabP, a *B. subtilis* KtrA homologue, and in this bacterium as well as in *L. lactis*, this second messenger was linked to central metabolism, since it allosterically regulates pyruvate carboxylase activity (81). c-di-AMP connection to central metabolism and the enzymes encoded in the *cdaAR glmM* operon are more detailed described for LAB (see below).

1.3.4. c-di-AMP and LAB

Even though LAB importance in basic and applied science is undeniable, only a few approaches with regard to c-di-AMP metabolism were done so far. The synthesizing enzyme CdaA is encoded in the widely conserved *cdaAR glmM* gene cluster, in which the gene for the regulatory protein CdaR is also present. The third gene of the operon codes for GlmM, an enzyme which converts glucosamine-6-phosphate to the peptidoglycan precursor glucosamine-1-phosphate, as previously described.

Different combinations of *L. lactis cdaA* operon genes expressed in *E. coli*, confirmed that CdaR and CdaA also interact in this bacterium and evidence suggests that CdaR would negatively regulate CdaA (82). Moreover, GlmM also modulates CdaA activity, connecting c-di-AMP metabolism to cell wall biosynthesis in *L. lactis* as well. Nevertheless, the exact mechanism remains unknown and, based on other model organisms, it is hypothesized that it would be through protein-protein interaction (82).

One of the first studies carried out in *L. lactis*, allowed the identification of spontaneous *gdpP* mutants after a high temperature incubation step. Among these mutants, heat-resistant and salt-hypersensitive phenotypes were observed, as well as enhanced growth in sub-lethal concentrations of penicillin G (74). Moreover, mutants in which GdpP activity is diminished manifest greater acid stress resistance, and this protein was also linked to the stringent response, since ppGpp exerts a strong inhibition on the c-di-AMP hydrolyzing activity of the DHH/DHHA1 domain (72, 83). Furthermore, it has been proposed that PAS domains act as heme sensors, since upon binding of this compound, GdpP is strongly inhibited (84). This would protect cells from heme toxicity, and in accordance to this, *L. lactis gdpP* mutant manifested increased sensitivity to heme group compared to the wild type (85).

On the other hand, it has been recently proved that pyruvate carboxylase (PC) enzyme from *E. faecalis* binds and is regulated by c-di-AMP. Interestingly, the binding site does not

seem to be conserved since *in silico* studies show that it is different from the c-di-AMP binding site of *L. monocytogenes* PC (86). This study was also extended to *L. lactis* and it was confirmed that c-di-AMP also binds to PC in this bacterium, being thus inhibited. Due to the lack of an α-ketoglutarate and glutamate dehydrogenase enzymes, and the presence of a non-functional isocitrate dehydrogenase, the TCA cycle is truncated in *L. lactis*. Consequently, it is proposed that the main function of PC is the de novo synthesis of aspartate via AspC. Very importantly, aspartate is a precursor for other amino acids, for pyrimidine and also for peptidoglycan cross-bridge amino acids. It is also theorized that PC is indirectly involved in osmoprotectant compounds synthesis, which is also one of the homeostasis mechanisms in which c-di-AMP is involved and would account for c-di-AMP interaction to a central metabolism enzyme (86).

c-di-AMP synthesizing and degrading activities were confirm in CdaA and GdpP homologous proteins of other LAB, like *Streptococcus suis* and *Streptococcus pyogenes* (87-89). RT-PCR studies showed in the last case that *gdpP* gene is expressed at least with two downstream genes, *rpl9* and *holB*. The former codes for the ribosomal protein L9, whereas the latter encodes the δ' subunit of DNA polymerase III (90). In the same work, a *gdpP* mutants study linked the lack of GdpP with increased resistance to the β-lactam antibiotic ampicillin, and the location of this enzyme inside the cell to the biosynthesis of the streptococcal virulence factor SpeB (secreted pyrogenic exotoxin B). Moreover, a *ΔgdpP* strain showed attenuated virulence in a murine model of subcutaneous infection.

In *S. pneumoniae* one CdaA homolog was also confirmed and as previously mentioned, two phosphodiesterases were identified, named Pde1 and Pde2. Pde1 is the GdpP homolog, whereas Pde2 is the shorter cytoplasmatic version described above (Fig. 3), and although both can degrade c-di-AMP, the cleavage products resulting from each are different: Pde1 cleaves c-di-AMP into pApA and Pde2 directly hydrolyzes it to AMP. Furthermore, Pde1 as well as Pde2 were shown to contribute to pneumococcal virulence, and even though Pde2 orthologs can be found in *B. subtilis*, *S. aureus* and *L. monocytogenes*, degradation of c-di-AMP by these enzymes has not been reported yet (56, 68).

Studies looking for c-di-AMP interaction partners in *S. pneumoniae* led to the identification of CabP, which belongs to the Trk/Ktr/HKT family of proteins and is the peripheral regulatory subunit of the potassium transporter SPD_0076. c-di-AMP binding to CabP would alter its conformation, disrupting the interaction between both parts of the K^+

uptake system, being thus inhibited (77). Interestingly, CabP interaction to c-di-AMP was also used to develop a c-di-AMP quantification method via ELISA (91).

In *S. mutans* a CdaA homologue was also identified, being able to synthesize c-di-AMP from ATP as well as ADP. Interestingly, a *cdaA* mutant was obtained in the same study, showing increased susceptibility to peptidoglycan-targeting antibiotics and oxidative stress, and also higher autolytic activity than the wild type strain (92, 93). In a different study it was also proved that c-di-AMP mediates biofilm formation of *S. mutans*, and high levels of the second messenger up-regulates expression of *gtfB*, an enzyme in charge of water-insoluble glucans production, critical for biofilm formation and virulence of this bacterium (94).

In the case of pathogenic bacteria, like the opportunistic LAB Group B *Streptococcus* (GBS), c-di-AMP can be secreted, activating consequently the Interferon-β (IFN-β) response of the host. Interestingly, *S. agalactiae*, belonging to GBS, keeps extracellular c-di-AMP levels low thanks to CdnP, an ectonucleotidase anchored to the cell wall. It is important to mention that c-di-AMP degradation is different from those previously described for phosphodiesterases, since CdnP acts sequentially with another ectonucleotidase, NudP, to degrade c-di-AMP into adenosine (95). In this way, over-activation of STING and its concomitant IFN-β induction is avoided and virulence is promoted.

Finally, in the opportunistic pathogen *E. faecalis* previously introduced, both classes of phosphodiesterases GdpP and PgpH where found encoded in its genome. Nevertheless, the obtaining of mutants involved in daptomycin resistance (an antibiotic targeting the cell membrane) only resulted in *gdpP* mutants, in agreement with previous research stating that this protein is more important for virulence than PgpH (57, 96). Initial mutations were also obtained in the *liaFSR* pathway, a three-component membrane stress response regulator, and it was observed that deletion of *liaR* lead to an increase in c-di-AMP intracellular levels. Even though the interaction between LiaFSR and c-di-AMP is not fully understood, the link between cellular wall damage and c-di-AMP levels was again established (96).

1.4. American Trypanosomiasis (Chagas disease)

American Trypanosomiasis (also known as Chagas disease) is a tropical disease caused by the flagellate protozoan parasite *Trypanosoma cruzi*. Even though the first cases were reported in the tropical areas of Latin America, according to the Health World Organization, an

estimate of 8 million people are infected worldwide with a cost of 10.000 deaths per year, affecting nowadays countries of North America, Europe, Asia and Australia as well (Fig. 4).

The life cycle of *T. cruzi* consists of four stages: the replicative epimastigote and amastigote stages and the infective non-replicative metacyclic and trypomastigote stages. It also requires a host of the Triatomine subfamily, most commonly known as kissing bugs, or Vinchucas in Spanish. When these bugs bite a mammal in order to feed from its blood, they normally defecate nearby and the metacyclic trypomastigotes residing in the faeces (which normally cannot penetrate the skin) can now enter the new host through the wound. After infection, they transform into amastigotes so they replicate inside the host cell and differentiate into trypomastigotes. Once the host cell disrupts, the trypomastigotes are released to the blood stream and are ready to infect new cells (97). There are also other less frequent routes of infection, such as blood transfusion, organ transplant and vertical transmission, i.e. 2-10% of infants are infected by their infected mothers. Horizontal transmission is possible as well, but more rare, since it implies mucosal penetration through the mouse or eyes (98).

Chagas disease manifests two clinical phases: the short acute phase of patent parasitemia, which is oligosymptomatic, and the chronic phase, which can remain asymptomatic for months or even years. The main symptoms in the chronic stage are related to heart failures and pathologies affecting organs of the gastrointestinal tract. For more than 40 years, Chagas disease treatment has been based on the nitroheterocyclic compounds nifurtimox (NFX; 3-methyl-4-[59-nitrofurfurylideneamine] tetrahydro-4H-1,4-tiazine-1,1-dioxide; developed by Bayer, Germany) and benznidazole (BZ; N-benzyl-2-nitroimidazole acetamide; RO7-1051; developed by Laboratorio Farmacéutico do Estado de Pernambuco (LAFEPE), Recife, Brazil and Laboratorio ELEA, Ciudad Autónoma de Buenos Aires, Argentina), which have trypanocidal activity against all parasitic forms. The main drawbacks of these drugs are: 1) their mechanisms of action are not entirely clear, 2) their side effects, which normally are the cause of treatment interruption and 3) the low efficacy, since during the acute phase only 50 to 80% of patients are cured and when used in the chronic phase, percentages drop to 8-20% (99, 100).

The World Health Organization (WHO) has established a set of characteristics that the ideal drug for Chagas disease treatment should gathered, namely: parasitological cure in both the acute and chronic phases, efficacy in one dose or a few doses, accessibility to affected population (low cost), absence of side effects or teratogenic effects, and no induction of

resistance. Unfortunately, there is no drug meeting all these requirements, and consequently new compounds are currently under investigation. Nonetheless, apart from a cure, priorities also fall into the improvement of diagnostics, health systems, vector control and disease prevention, i.e. the development of vaccines.

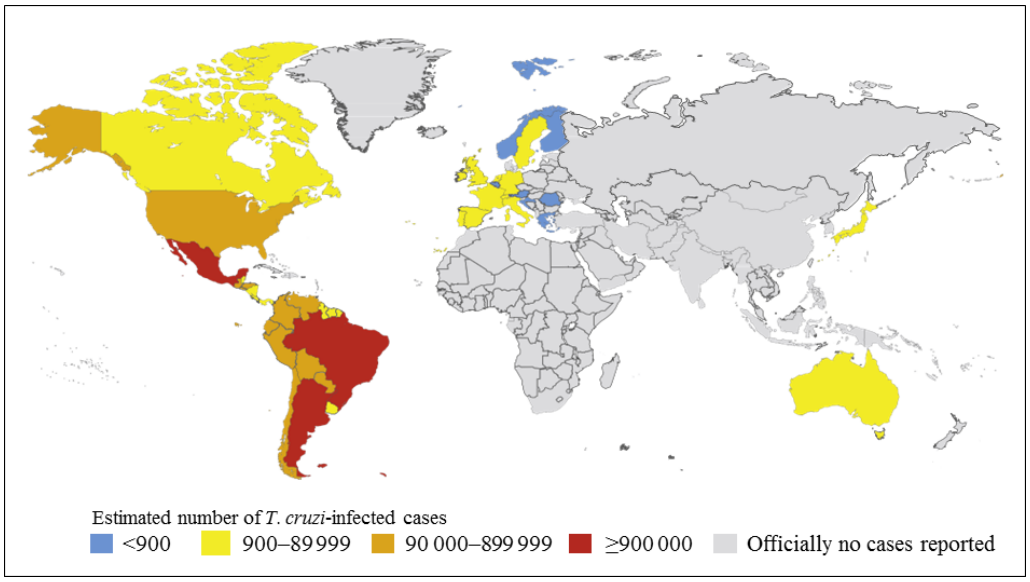

Fig. 4 Global distribution of Chagas Disease cases. Even though it is a tropical disease which originally occurred in South America, it is nowadays distributed in the four continents represented in the picture. Numbers are based on official estimates 2006 – 2010. Adapted from Trends in Parasitology.

1.4.1. Chagas prevention

Nowadays, several investigations are directed towards the formulation of vaccines using as antigens key peptides or proteins involved in infection establishment. In this sense, one of the main candidates is the *T. cruzi* trans-sialidase protein (TS) (101, 102). This is an enzyme of 160-200 kDa, which is anchored to the outer cellular membrane by glycosylphosphatidylinositol (GPI). It catalyzes the transfer of sialic acid from glycoproteins of the host to the β-Gal residues on mucine proteins covering the parasite membrane. In this way, *T. cruzi* trypomastigotes make their way into new cells upon establishment of infection, and they mask recognition sites for the immune system, often deriving in auto-immune responses. TS also helps the parasite in recognizing host cells and attaching to its sialic acids and/or β-Gal residues of their surface, through TS active site or other domains (103).

TS synthesis depends on a gene superfamily, which resulting proteins range from 60 to more than 200 kDa. Of this superfamily, only 12 are active, and they vary by their level of

expression in the different stages of the parasite life cycle, their degree of glycosylation and the SAPA domain (shed acute phase antigen) which are tandem repeats of a 12 amino acids sequence. Since mammals lack TS and it is essential for the parasite because it cannot synthesize sialic acid *the novo*, this protein is currently under study as a good candidate for vaccine formulations. Different antigens have been developed based on this protein, and promising results were obtained (104).

For example, antigens TSA-1 (Trypomastigote surface antigen-1) and Tc24 (flagellar calcium binding protein of 24 kDa) have been thoroughly investigated and are highly immunogenic. Moreover, the protective efficacy of both has been proved in mice models and it has been reported as well that an immunotherapeutic DNA vaccine could control Chagas disease, reducing parasitemia and cardiac tissue damage (105, 106). Very recently, studies suggested the presence of antigen-specific memory cells induced by natural infection in seropositive patients and, in the same work, a long lasting humoral and cellular response to these antigens was evidenced, even in subjects on the chronic phase, after 10 years of infection (107).

1.5. Subunit vaccines

The first world-wide spread vaccine was against smallpox in the 18th century. Upon the massive outbreak, it was noticed that infected people who survived the disease were immune. Based on this observation, a common practice of subcutaneous inoculation of smallpox virus into non-immune individuals was started. The virus was obtained from pustules of an infected person, and no regulations or thorough investigations existed for vaccination, for what considerable health risks existed, even though mortality caused by smallpox itself was significantly higher. A percentage of vaccinated patients died or contracted another disease, like tuberculosis or syphilis due to the lack of good hygiene conditions. Nevertheless, vaccination against smallpox was considered a success (108).

During the 19th century, many others vaccines were developed, with similar strategies. For example, rabies virus extracted from dead infected rabbits were weakened by drying and then used as inocula, and killed whole cells of *Vibrio cholerae* were the immunization strategy against cholera (109, 110). Like in the former example, attenuated pathogens by drying, boiling or propagation through a foreign host are the base of “attenuated vaccines”. The principal advantages of these vaccines are their low cost and the fact that they trigger a strong, long-

lasting immune response. Nevertheless, since the live pathogen is administered, the possibility exists that it can regain its virulence, and special attention needs to be paid with immunocompromised patients. Moreover, these vaccines need to be refrigerated, so their transportation and application in poor areas is an issue (111).

An alternative to attenuated vaccines are toxoid vaccines, which do not make use of the pathogen at all, and are the strategy of choice for vaccine development when the disease is caused by a toxin synthesized by the pathogen. Such is the case of tetanus, which is caused by a toxin secreted by *Clostridium tetani* and after formaldehyde treatment, minor molecular changes occur and it is converted to a toxoid. The purified product is a non-toxigenic compound capable of stimulating the immune system to generate specific antibodies that, in the event of a *C. tetani* infection, bind to the toxin. This complex is not able to bind to the regular toxin receptor binding sites, so the development of the disease is prevented (112). The main benefit of these vaccines is the fact that there is no risk of disease nor to revert to a virulent form. Moreover, the compounds are normally stable and less susceptible to temperatures, light and humidity. Nevertheless, they normally need an adjuvant (see below) and this usually causes local reactions at the vaccination site.

Another class of vaccines are the inactivated vaccines, like the case of hepatitis A vaccine, in which the pathogen is heated or chemically treated so that its capacity of replication is lost, but the antigens are still recognized by the immune system. The adaptive immune response is very similar to that of a toxoid vaccine, although inactivated vaccines have the advantage of having all antigens participating in the infection and therefore a broader range of antibodies is synthesized. Nevertheless, among the drawbacks of these vaccines it can be mentioned that one dose normally is not enough to trigger a strong signal to the adaptive immune system, so they are usually given in several doses and, like toxoid vaccines need to be adjuvanted (see below) (112).

Finally, the latest development in vaccines are the subunit vaccines. In this case, a particular antigen or set of antigens are provided so they are recognized by the immune system and, consequently, specific B cells are produced. Subunit vaccines can be further divided into subgroups depending on whether the antigen is a peptidic compound or a polysaccharide, which will define the kind of immune response they will generate in the patient (112). If the antigen derives from a protein (such is the case of hepatitis B and influenza vaccines) then a T-dependent response will be trigger, like for the case of toxoid vaccines. In the case of

polysaccharide derived antigens, the response will be T-independent (e.g. the pneumococcal vaccine Pneumovax) (113). Advantages and disadvantages are similar to those of the toxoid vaccines, except that with the advances of molecular biology, strategic antigens (and adjuvants) can be designed and combined to widen the spectra of pathogen strains against which the vaccine is active (114).

1.5.1. *L. lactis* in the industry: its potential as delivery vector

In the last decades, a considerable amount of research has been prompted to use food grade bacteria as live vaccines. The starting point to consider a bacterium suitable for antigen delivery is its innocuity. In this sense, *L. lactis* classification as GRAS and QPS as previously mentioned, makes it appropriate as regards safety. *L. lactis* can also survive its passage through the gastrointestinal tract (GIT) for 2-3 days, without colonizing the mucosal surface of the host (115). Moreover, due to its lack of lipopolysaccharide attached to their cell membrane, it does not stimulate the host immune response.

This species in particular has considerable importance in medicine and the pharmaceutical industry, and it was in fact, the first genetically modified microorganism for the treatment of a human disease (116). For an outline with examples supporting *L. lactis* use in food and pharmaceutical industries, see Table I.

In 1989 Iwaki et al. proposed for the first time the use of *L. lactis* for the development of a mucosal vaccine against caries by use of a strain expressing a surface *Enterococcus mutans* protein. When the killed recombinant bacteria were orally administered in mice, salivary IgA and serum IgG response to the protein were induced (117).

Nowadays, many investigations see the use of *L. lactis* as an opportunity to develop competent treatments for diseases and conditions currently without a cure. For example, Inflammatory bowel diseases (IBD) like Chron's disease and ulcerative colitis are in the urgent need for the formulation of an efficient and cost effective drug treatment. Even though their causes are not completely understood, inflammation of GIT is a common consequence. The first human trial with a therapeutic bacterium, genetically engineered to treat GIT inflammation was conducted in 2006. In this work, an *L. lactis* secreting interleukin-10 strain gave interesting immunological results and most importantly, it was demonstrated to be safe for humans (118).

L. lactis is therefore seen as one of the best options to deliver any molecule at the mucosa level, able to alleviate symptoms and provide the patient with a better life quality (119).

Other investigations were also developed using *L. lactis* as a recombinant live delivery vector, like the study performed with the tetanus toxin fragment C (TTFC). Bacteria expressing TTFC anchored to the cellular membrane were subcutaneously administered in mice, and antibody production was proven to protect mice against the lethal infection. Nasal and oral administration showed similar protection efficacies. In the former, the antigen was intracellularly overexpressed and IgG antigen production in serum protected 75% of mice (120). Nevertheless, for this example, the expression system was based on *E. coli* T7 bacteriophage promoter, and although it allows high expression levels of heterologous proteins of interest, it is not suitable for the final model of oral immunization. Since 2000 this system has been replaced by the nisin inducible system NICE previously described.

Since the first reported investigations about *L. lactis* as a delivery vector were made, several others were carried using this methodology for different antigens able to elicit an immune response. Mice orally immunized with recombinant *L. lactis* secreting *Brucella abortus* superoxide dismutase were reported to develop systemic and mucosal SOD-specific immune responses (121). Moreover, an oral live vaccine expressing an attenuated version of the Staphylococcal enterotoxin type B (SEB) was also reported to elicit immune protection. In this study, both intracellular and secreted versions of antigen production in the intestinal mucosa of mice showed to stimulate production of specific IgG and IgA antibodies in the serum and the feces, respectively (122). Production of specific IgA in intestinal mucosa was also obtained when BALB/c mice were orally immunized with a strain of *L. lactis* constitutively expressing EspB, a key protein involved in the attachment of enterohemorrhagic *E. coli* O157-H7 (EHEC) to enterocytes (123). Also, a vaccine against *Rhodococcus equi*, the main causal agent of pneumonia in foals, was developed using an *L. lactis* recombinant strain secreting the virulence associated protein VapA. In this study, intragastric and intranasal routes were considered, obtaining both humoral and cell-mediated immune responses (124).

As it can be appreciated, the use of *L. lactis* as a delivery vector of antigens synthesized in the cytosol, presented on the cellular membrane and secreted to the extracellular space, is a recently developed technology currently being exploited. Apart from its applications against bacterial pathogens of humans and animals with industrial relevance, interesting results were also obtained against the human papillomavirus and rotavirus, the most common cause of severe

dehydrating diarrhea in children (125, 126). When genetically modified LAB are administered in mucosal tissues, both mucosal and systemic immune responses are triggered. Therefore, due to its non-invasive application and its rather simple manipulation without the need of highly qualified personnel, mucosal vaccines are gaining a central position in new vaccine prototypes formulation. Their production would also imply a reduction in costs, since the live microorganism synthesizes and delivers the antigen compound of interest, without the need of purification (127).

Table I. Examples of *L. lactis* strains and strategies with potential use in food and pharmaceutical industries

Parental Strain	**Strategy**	**Product**	**Location**	**Application**	**Reference**
NZ3950	NICE system	L-alanine	C	Food sweetener	Hols et al. 1999 (128)
NZ9000	Spontaneous mutant	Vitamin B11	C	Nutritional supplement	Sybesma et al, 2003(129)
NZ9000	Spontaneous mutants / NICE system	Vitamin B2	C	"Vitamin factory"	Burgess et al. 2004 (130)
IL1403	pILPtuf Promoter / Usp45-secretion signal peptide	Interleukin -6	S	Adjuvant	Li et al. 2015 (131)
Not informed	pUB1000 / Usp45-secretion signal peptide	Insulin analogue	S	Drug delivery	Agarwal et al. 2014 (132)
MG1363	pTREX	Anti-TNF a nanobodies	S	Bowel disease treatment	Vandenbroucke et al. 2010 (119)
NZ9000	NICE system	Antigen	S C A	Rotavirus vaccine	Marelli et al. 2011
IL1403	pILPtuf promoter / USp45- secretion signal peptide	Adjuvant	S	Cancer vaccine	Kim et al. 2015 (133)
MG1363	P170 promoter	Allergen	S	Allergy	Glenting et al. 2007 (134)
NZ9000	pSEC:LEISS	Carcinoembryonic antigen (CEA)	C A	Cancer vaccine	Xiaowei et al. 2016 (135)

C: cytoplasmic – S: secreted – A: cell wall anchored

1.5.2. c-di-AMP as Adjuvant

A key factor for the development of human subunit vaccines is not only to define a suitable antigen but also to select an adequate adjuvant. Vaccine adjuvants are compounds added to vaccine formulations that enhance the immunogenicity of antigens *in vivo*. They are further categorized based on their mechanisms of action, being divided into delivery systems (or particulate adjuvants) and immune potentiators (or immune stimulators). The former enhance the innate immune response either directly or through pattern-recognition receptors, whereas the latter help with antigen presentation and localization (136). Several advantages can be mentioned when describing the scope of adjuvants use, namely: stronger and longer immune response (especially important for weakened immune system population), improved antibody production against a broader spectrum of pathogenic strains, mucosal immune response induction, and increased biological half-life of vaccines. Hence, the amount of antigen needed is lowered, as well as the number of doses required for proper immune response generation, which consequently lowers costs of vaccination programs as well (137).

Cyclic-di-nucleotides (CDNs) have recently emerged as pathogen associated molecular patterns (PAMPs) and effective adjuvants. PAMPs and adjuvants are specifically important for the mucosal route, when antigens suffer mechanical removal and structural modifications by pH changes and enzymatic degradation. Particularly 3´5´-cyclic-di-adenosine monophosphate (c-di-AMP) was demonstrated to be a suitable candidate for vaccine adjuvant, since it has showed the capacity to activate *in vitro* dendritic cells of murine and human origin, one of the most important steps to stimulate the adaptive immune response. Several investigations have been carried out to test this second messenger action as an adjuvant when co-administered with an antigen of interest. Results after intra-nasal and oral immunization showed improved stimulation of immune responses at both systemic and local levels. Moreover, *in vivo* experiments also evidenced that c-di-AMP can promote MHC class I restricted immune response by $CD8^+$ T cells, promote specific IgG and IgA production and enhance cellular proliferative responses (138). This consequently triggers the stimulation of Th1, Th2 and Th17 cells, as well as the induction of cytotoxic T lymphocytes. Moreover, it was recently demonstrated not only that c-di-AMP triggers a type I IFN response, but also that it down-regulates it in the inductive phase of an adaptive immune response, which is a positive characteristic for its use in vaccines (139).

In a proposed intranasal vaccine model for Chaga's disease, where c-di-AMP was co-administered with antigen TC52 (essential for the parasite *T. cruzi*), high specific Tc52-specific IgA titers were found in nasal lavages. Importantly, this results were significantly different from those obtained in the absence of the adjuvant c-di-AMP, and only in its presence, stronger cellular and humoral immune responses were induced (140). In another vaccine prototype based on archeaosomes as delivery system of a hepatitis C virus antigen, a robust T cell immune response was observed when adjuvanted with c-di-AMP (141). The same study also showed that a partial intranasal immunization regimen also triggers the immune response, opening the range of possibilities as regards administration.

Another non-invasive and economically favorable regimen of vaccine administration is the sublingual route. As with the intranasal, the main advantages are the simple immunization protocol along with its consequent possibility of being handled by personnel with no advanced training, which would allowed massive administration. This would provide an important benefit for example, during an epidemic outbreak. In this regard, a virosome-based vaccine against influenza virus H5N1 was tested using c-di-AMP as adjuvant, and the sublingual and intranasal inoculation as routes of administration, evidencing strong humoral and cellular responses (142).

In conclusion, the formulation of mucosal, live, subunits vaccines adjuvanted with c-di-AMP and with food grade bacteria as delivery systems proposes an innovative paradigm currently under investigation.

1.6. Objectives

Under the theoretical framework developed, the general objective of this thesis is to broaden the knowledge of c-di-AMP metabolism in firmicutes, and specifically in the Lactic Acid Bacteria *Lactococcus lactis* and *Enterococcus faecalis*. In addition, the aim of this work is to approach research from two perspectives: the importance of basic investigation around this newly discovered second messenger, as well as the vast application spectra it offers to the biotechnological and pharmaceutical industries.

Specific objectives set to accomplish the aforementioned general objectives are:

i) To identify and characterize c-di-AMP interaction partners in *L. lactis*

ii) To develop engineered strains able to modify c-d-AMP levels in *L. lactis* and *E. faecalis* and study their physiological impact

iii) To formulate a vaccine prototype by use of an *L. lactis* strains producing high levels of c-di-AMP

iv) To perform a first approach into the virulence of GdpP in *E. faecalis*

2. Materials and methods

"An experiment is a question which science poses to Nature, and a measurement is the recording of Nature´s answer" Max Planck

2.1. Materials

Lists of chemicals, commercial kits, enzymes and oligonucleotides used in this work are presented in the appendix.

2.2. Strains and plasmids

Bacterial strains and plasmids constructed for this work are detailed in the appendix.

2.3. Methods

2.3.1. General methods

General methods routinely used in this work are mentioned in Table II

Table II. General methods

Method	Reference
Absorption measurement	Sambrook *et al*. 1989 (143)
DNA gel electrophoresis	Sambrook *et al*. 1989 (143)
Plasmid preparation from *E. coli*	Sambrook *et al*. 1989 (143)
Ligation of DNA fragments	Sambrook *et al*. 1989 (143)
Protein quantification	Bradford 1976 (144) - Lowry 1951 (145)
Protein gel electrophoresis (denaturing)	Laemmli 1970 (146)
Sequencing according to the chain termination method	Sanger *et al.* 1977 (147)

2.3.2. Storage and bacterial growth

E. coli DH5α and BL21 were grown in Luria Bertani (LB) medium at 37 ºC in aerobic conditions with vigorous shaking. *E. coli* LB650 and 2003 were grown under similar conditions in LB with addition of 200 mM KCl or in M9/M9mod (see Table III) supplemented with a suitable carbon source and 50 mM KCl, unless otherwise stated.

LAB were cultivated in M17 medium supplemented with 0.5% glucose (M17G), in aerobic conditions without shaking. Incubation temperatures were 30 ºC and 37 ºC for *L. lactis* and *E. faecalis*, respectively.

In all cases -80 ºC stocks prepared in 10% glycerol were propagated twice to obtain a final overnight (ON) saturated culture. This was used in turn to inoculate fresh media at an initial optical density (OD_i) of 0.05.

Media composition

Media composition is detailed in Table III. pH was adjusted to 7.0 or 5.5 with NaOH or HCl according to the experiment. In all cases, deionized water was used, and media were autoclaved for 20 minutes at 121 ºC and 2 bar. Thermo-labile compounds were separately prepared, filter-sterilized and stored at their optimal temperature. For agar plates, 1.5 % agar was added.

Table III. Growth media composition

Medium	**Component**	**Quantity**
Luria Bertani (LB) (143)	Casein Peptone	10 g
	Yeast extract	5 g
	NaCl	10 g
	dH_2O	Add to 1 Lt
M17 (Terzaghi and Sandine, 1975) (148)	Bactopeptone	10 g
	Beef Extract	5 g
	Yeast extract	2.5 g
	Ascorbic Acid	2.5 g
	Sodium β-glycerophosphate	19 g
	$MgCl_2$ [*1]	5 mM
	$MnCl_2$ [*1]	1.6 mM
	dH_2O	Add to 1 Lt
M9 [*2] (Sambrook and Russell, 2001) (149)	M9 base 20X	50 ml
	$MgSO_4$ 1M	1 ml
	$CaCl_2$ 0.1 M	1 ml
	$FeCl_3$ 1 mM	0.5 ml
	Carbon source	1 %
	Casaminoacids10 %	66 ml
	Proline 4 M	10 ml
	Thiamine 1 M	1 ml
	dH_2O	Add to 1 Lt
	$Na_2HPO_4.2H_2O$	140 g

M9 base medium	KH_2PO_4	60 g
	NH_4Cl	20 g
	dH_2O	Add to 1 Lt

*[1] Added from 1M solutions, after autoclave. *[2] In M9mod medium, KH_2PO_4 is replaced for equimolar quantities of NaH_2PO_4.

When needed, growth media were supplemented with antibiotics as selective agents (Table IV), which were prepared in 1000-fold concentrated stock solutions. Deionized water was used for dissolution, except for erythromycin and chloramphenicol, in which cases ethanol was employed. Sterilization was performed by filtration and storage at -20 ºC. Antibiotics were added to media before inoculation.

Table IV. Antibiotics

Bacterium	**Antibiotic**	**Concentration**
E. coli	Ampicillin	100 mg/ml
	Erythromycin	150 mg/ml
	Chloramphenicol	30 mg/ml
	Kanamycin	40 mg/ml[1]
		50 mg/ml[2]
L. lactis and *E. faecalis*	Erythromycin	5 mg/ml
	Chloramphenicol	10 mg/ml

[1] *E. coli* EC101 [2] *E. coli* LB650

For expression or gene deletion experiments, inducers and indicators were respectively added to media (Table V).

Table V. Inducers and indicators

Compound	**Stock Solution**
Arabinose	5 % p/v
IPTG	0.5 M
Nisin	50 µg/ml
X-Gal	40 mg/ml in (DMF)

2.3.3. *E. coli* electroporation

E. coli electrocompetent cell preparation

5 ml of a saturated ON culture of the *E. coli* strain of interest were used to inoculate 500 ml of LB medium at an OD_i of 0.05. Cells were incubated in a 2 Lt. Erlenmeyer at 37 °C with vigorous shaking until OD reached 0.50. The culture was then cooled in ice for 20 min and centrifuged for 5 min at 5000 rpm and 4 °C. Two wash steps were performed afterwards with 500 ml ice-cold $_dH_2O$ and one with 10 ml glycerol 10%. Finally, the pellet was resuspended in 1 ml glycerol 10% and separated in 100 µl aliquots. Samples were stored -80 °C.

Electroporation

Cells were electroporated as described in Dornan and Collins, 1990, under the following conditions: 25 µF, 2,5 kV y 200 Ω. After the pulse, 1 ml of LB was added to the electroporation cuvette and samples were incubated for 1 hour at the 37 ºC. Samples were plated in LB supplemented with the corresponding antibiotics and incubated ON.

2.3.4. LAB electroporation

LAB electrocompetent cell preparation

Strains of LAB under study were grown in M17G supplemented with 0.5 M saccharose (M17SG) and 1% glycine. The ON saturated culture was diluted 50 times in fresh M17SG + 1% glycine. Cells were incubated at 30 ºC or 37 ºC for *L. lactis* or *E. faecalis*, respectively until OD reached 0.4-0.6. The culture was then harvested for 5 minutes at 5000 rpm at 4 ºC. Pellets were washed in 0.5 M ice cold saccharose + 10% glycerol using half of the culture volume. Centrifugation was performed at 6000 g for 8 minutes at 4 ºC. Cells were resuspended in 0.5 M ice cold saccharose + 10% glycerol using 1/200 of the initial culture volume.

Electroporation

LAB were electroporated as described in Dornan and Collins, 1990, under the following conditions: 25 µF, 2,5 kV y 200 Ω. After the pulse, 1 ml of M17SG was added to the electroporation cuvette and samples were incubated for 1.5 hours at the optimal growth temperature of the strain under study (30 ºC for *L. lactis* and 37 ºC for *E. faecalis*). Cells were plated in M17SG supplemented with the corresponding antibiotics and incubated ON or until colonies appeared.

2.3.5. DNA manipulation

DNA extraction

For plasmidic DNA extraction from *E. coli*, 3 ml of a saturated ON culture was harvested at 4500 rpm for 5 minutes. The pellet was then resuspended and processed using the Wizard® Kit (Promega) or the NucleoSpin® Plasmid Kit (Macherey-Nagel), according to manufacturer's instructions. Elution was performed with pre-warmed $_{d}H_2O$ at 70 ºC.

Genomic DNA was similarly prepared, using the Wizard® Genomic DNA Purification Kit (Promega) or the NucleoSpin® Microbial DNA Kit (Macherey-Nagel).

When working with Gram-positive bacteria, 10 ml of an ON saturated culture were harvested and the pellet was incubated with 10 mg/ml lysozyme at 30 ºC for 30 minutes. The protocol was then completed using plasmidic or genomic DNA extraction kits.

Polymerase Chain Reaction (PCR)

Reactions were prepared in final volumes of 25 or 50 µl, using 0.2 µM of specific primers and 0.5 µM of dNTPs. Pfu, Taq or Phusion polymerase were used according to manufacturer's instructions. PCRs were performed in a thermocycler with 30 cycles of denaturation, annealing and elongation (Table VI). 0.5 µl of plasmidic DNA or of a 10-fold dilution of genomic DNA were used as template.

Oligonucleotides were designed using VectorNTI (Invitrogen) or Geneious 7.0.2 (Biomatters) softwares and purchased from Sigma-Aldrich, Genbiotech or Invitrogen. A list of primers used in this work is provided in the appendix (Table XVII).

Table VI. PCR steps

Step	Temperature (ºC)	Time
Initial denaturation	98.0	5'
Denaturation	98.0	1'
Annealing	52.0 – 59.0*[1]	45''
Elongation	72.0	Variable*[2]
Final elongation	72.0	10'
Hold	4.0	hold

*[1] According to primer melting temperature (5 degrees lower)
*[2] According to enzyme processivity and product length

DNA electrophoresis

DNA electrophoresis was performed according to Sambrook et al., 1989. Horizontal gels were prepared in TAE buffer (Table XVIII) with agarose concentrations ranging from 0.8 to 1.2 % according to DNA fragment size. For DNA visualization, 1X GelGreen (Biotium) or HD Green Plus DNA stain (INTAS Science Imaging Instruments GmbH) was added.

Gels were submerged in TAE, samples previously stained in 1X sample buffer (0,25 % (p/v) bromophenol blue, 0,25 % (p/v) xilenecyanol and 30 % (p/v) glycerol) were loaded and run at 100 mA. DNA from λ phage digested with *Eco*RI and *Hind*III enzymes (PBL Productos Bio-lógicos) was loaded as reference and DNA bands were visualized using a blue-light or UV transilluminator.

DNA purification

Purification of linear DNA fragments after PCR or enzymatic digestion was performed using the QIAquick® PCR purification kit (QIAGEN, Hilden), or the Gel Band Purification Kit (GE Healthcare Life Sciences) when purifying DNA from agarose gels. Protocol was followed as specified by manufacturers and final elutions were done in pre-warmed $_{d}H_2O$ at 70 ºC.

DNA enzymatic digestion

For DNA digestion with restriction enzymes, the protocol of Sambrook (1989) was generally followed. Fast Digest or regular endonucleases purchased from Thermo scientific were used. When linearizing plasmids, dephosphorylation was performed using Calf Intestinal Alkaline Phosphatase (CIAP, Promega) or FastAP Alkaline Phosphatase (Thermoscientific). In all cases, reactions were performed following manufacturer's instructions.

Ligation of DNA fragments

Ligase enzyme was acquired from Takara or Thermoscientific and reactions were prepared in final volumes of 20 µl. Manufacturer's protocols were followed, using in general 100 ng of vector and a 1:5 molar ratio to calculate the volume of the fragment of interest. When ligating two DNA fragments, equimolar quantities were used. Before electroporation, ligase enzyme was heat-inactivated.

Clone screening

In order to screen for positive clones, 3 ml of LB (10 ml of M17G in the case of LAB) supplemented with the corresponding antibiotics were inoculated with a putative positive colony (normally 12 colonies were analyzed) to obtain an ON saturated culture. 50 µl of these samples were harvested at room temperature at 5000 rpm for 5 minutes. Pellets were resuspended in 25 µl of EDTA 10 mM pH 8.0 and cells were disrupted by addition of 25 µl of freshly prepared crack buffer (Table VII). Samples were incubated for 5 minutes at 70 ºC and cooled in ice afterwards. Next, 2 µl of crack dye were added (Table VII). After 10 minutes of incubation in ice, samples were centrifuged for 5 minutes at 12000 rpm and 4 ºC, and they were finally analyzed by agarose electrophoresis. Plasmids with inserts of interest were evidenced by its differential migration in the gel when compared to the empty vector.

Table VII. Clone screening solutions

Solution	Composition	Concentration
Crack buffer	Sucrose	0.2 % p/v
	SDS	0.5 %
	NaOH	0.2 N
Crack dye	KCl	3 M
	Loading dye	1X

DNA Sequencing

Plasmids and fragments of interest were sent to the DNA sequencing facility at the University of Maine, or to SeqLab Sequence Laboratories, Göttingen GmbH.

2.3.6. Gene deletion

After electroporation with pBVGh vector (Table XVI), cells were incubated at 30°C in M17G Em^5 X-Gal 100 µg/ml agar plates.

A single blue colony was used to inoculate fresh M17G + 5 µg/ml Em, which was then incubated ON at 42 °C. This culture was diluted 1/100 in fresh media, and it was incubated again at 42 °C ON.

Then, cells were propagated in fresh media without antibiotic, inoculating 0.05 % of the culture volume. After inoculation, samples were incubated 4 hours at 30 °C and ON at 42 °C. This step was repeated twice.

Serial dilutions (normally 10^{-4}; 10^{-5}; 10^{-6}) were plated in medium with 100 µg/ml X-Gal and no antibiotic. Plates were incubated at 42 °C for 24-48 hours, or until colonies were distinguished.

White colonies were restricked in plates with and without antibiotic, and those which were sensitive were checked by PCR using a pair of primers hybridizing in the adjacent up and downstream genes. A set of primers amplifying an internal sequence of the gene was also used as negative control.

2.3.7. Protein preparation and analysis

Cell disruption by French Press

The cell body and the piston were kept in ice prior to use (French Pressure Cell, Thermo Scientific). Samples were also cooled and washed in buffer W (Table XVIII). Cell pellets were disrupted once when working with *E. coli* and three times when working with *L. lactis*. Pressure was 18000 psi in all cases.

Cell disruption by Tissue Lyser

5 ml of a LAB culture of interest were harvested for 10 minutes at 5500 rpm. Cells were washed in 1 ml of buffer W. Next, pellets were resuspended in lysis buffer and transferred into a 2 ml screw-cap microvial containing 0.5 g of glass beads (0.1 µm). Cell walls were disrupted using a mini-beadbeater-16 (Biospec, USA) in 3 cycles of 5 minutes, incubating the samples in ice for 3 minutes in each interval. Crude extracts were then processed according to the desired protocol. For larger samples, 4 ml of sample were similarly disrupted in a 5 ml screw-cap microvial using 0.8 g of glass beads. Buffers are listed in Table XVIII.

Expression test of heterologous proteins in *E. coli*

10 ml of a suitable medium were inoculated with the *E. coli* strains under study. When OD_{600} reached 0.5, 0.5 mM IPTG were added and cultures were incubated for three more hours

at 37 ºC and continuous vigorous shaking. Samples were normalized in 100 µl with equal cell amounts and spinned down in a table top centrifuge. Pellets were resuspended in 15 µl of SDS loading dye and boiled for 5 minutes at 95 ºC. Finally, the whole volume was loaded in an SDS-PAGE gel for expression analysis.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis was based on Laemmli (1970) protocol. 1.0 mm polyacrylamide gels with concentrations ranging from 10 – 15 % were prepared according to the molecular size of the proteins under study (Table VIII). Samples were boiled at 95 ºC for 5 minutes in sample buffer. Normally, 30 µg of total protein were loaded in the gel, which was submerged in running buffer. Electrophoresis was performed at room temperature, at current intensity of 20 mA. Buffers are listed in Table XVIII.

PageRulerTM Plus Prestained Protein Ladder (ThermoFischer Scientific) or unstained Low Range Molecular Marker (BioRad) were used as size standards.

Finally, gels were analyzed by Coomassie staining, silver staining or Western Blot.

Table VIII. SDS-PAGE Gel composition

Solution	**Separating gel**			**Stacking gel**
Acrylamide (Stock 30 % acrylamide-bisacrylamide 19:1)	10 %	12 %	15 %	5 %
Tris-HCl (Stock 1.5 M)	375 mM[*1]			187.5 mM[*2]
SDS (Stock 20 %)	0.1 %			
APS (Stock 10 %)	0.1 %			
TEMED	0.04 % (v/v)			0.1 % (v/v)

[*1] Tris HCl pH 8.8 [*2] Tris HCl pH 6.8

Coomassie Staining

For Coomassie staining after SDS-PAGE, gels were incubated in Coomassie R-250 for 30 minutes at room temperature in a rocking shaker. For protein visualization, gels were afterwards boiled or incubated in destaining solution containing 20 % v/v ethanol and 5 % v/v acetic acid until protein bands were evidenced.

Silver staining

When protein amounts were low, visualization was accomplished by silver staining which allows a more sensitive detection (up to 1 ng of protein) (150). For this, the following protocol was followed:

- Fixation: the gel was incubated in fixing solution, at least during 1 hour
- Wash: 3 times with 50 % ethanol, during 20 minutes
- Reduction: Performed in 0.02 % (p/v) $Na_2S_2O_3$
- Wash: 3 times with dH_2O for 20 seconds
- Impregnation: the gel was incubated for 15'-25' in impregnating solution
- Wash: 3 times with dH_2O for 20 seconds
- Development: developing solution was used until bands appeared
- Wash: 3 times with dH_2O for 20 seconds
- Development was stopped by incubation in stopping solution

Table IX. Silver staining solutions

Solution	Composition	Concentration
Fixing	Methanol (100 %)	50 % (v/v)
	Acetic acid (100 %)	12 % (v/v)
	Formaldehyde (37 %)	0.1 % (v/v)
Impregnating	$AgNO_3$	0.2 % (p/v)
	Formaldehyde (37 %)	0.037 % (v/v)
Developing	Na_2CO_3	12 % (p/v)
	$Na_2S_2O_3$	0.0004 % (p/v)
	Formaldehyde (37 %)	0.05 % (v/v)
STOP	Na_2-EDTA	1.86 % (p/v)

Western blot

After running an SDS-PAGE, the gel was incubated in transfer buffer (Table X) for 5 minutes. Proteins were then transferred to a nitrocellulose membrane (Schleicher & Schuell BA85, 0,45 µm) using a mini-protean two cell unit (Bio-Rad, Hercules, CA, USA) (the sponges, Whatman paper and the membrane were also previously incubated in transfer buffer). Protein transfer was performed at 100 mA for 2 hours or at 20 mA for 12 hours, and efficiency was assessed by staining with Pounceau Red S (Sigma, USA). After this, the membrane was treated according to the following protocol:

- Wash: Rinse with dH_2O and incubate twice in TBS for 10 minutes
- Block: Incubate 1 hour in TBS + 5 % milk
- Wash: Twice in TBS + 0.05 % Tween and 0.2 % Triton for 10' minutes
- Wash: Once in TBS for 10 minutes
- Incubate 1 hour with Anti-His (1/200) in TBS + 5 % milk
- Wash: Twice in TBS + 0.05 % Tween and 0.2% Triton for 10 minutes
- Wash: Once in TBS for 10 minutes
- Incubate 1 hour with secondary antibody in TBS + 5 % milk
- Wash: Four times in TBS + 0,05 % Tween + 0,2 % Triton for 10 minutes
- Wash: Once with phosphatase buffer for 10 minutes
- Incubate in the dark in 10 ml phosphatase buffer + 33 µl 5-bromo-4-chloro-3-indoyl phosphate (BCIP; 50 mg/ml in 100 % DMF) + 66 µl *p*-nitroblue tetrazolium chloride (NBT; 50 mg/ml en 70 % DMF)

Table X. Western Blot Solutions

Solution	Composition	Concentration
Transfer	Methanol (100 %) Tris Glycine	20 % (v/v) 20 mM 150 mM
TBS 10X	Tris NaCl Adjust pH to 7.5	50 mM 150 mM
Phosphatase buffer	Tris NaCl $MgCl_2$ Adjust pH to 7.5	100 mM 100 mM 10 mM

2.3.8. Microplate growth curves

All microplate growth curves were performed in a final volume of 200 µl. ON saturated cultures were used to inoculate fresh media at an OD_i of 0.05. When OD_{600} reached 0.5, cells were harvested and resuspended to inoculate microplates at an OD_i of 0.05. Reads were performed every 15 minutes for 16 hours, at the optimal growth temperature of the microorganism under study.

Growth curves on minimal salt media

When curves were performed in minimal salt M9mod media, strains derived from *E. coli* LB650 were propagated twice from -80 ºC stocks in M9mod supplemented with 50 mM KCl and 1 % glucose. The ON saturated culture thus obtained was used to inoculate fresh M9mod media supplemented with 10 μM IPTG and 0.1 mM KCl for strains *E. coli* LB09 and LB10, 50 mM KCl for strain LB11 and 100 mM KCl for LB08 and LB12. When OD reached 0.5, cultures were harvested and incubated for 1 hour in the initial volume of fresh media, this time without KCl supplementation. Afterwards, cells were harvested and washed three times with fresh media with no KCl. These samples were then used to inoculate fresh M9mod media in microplates supplemented with 1 % glucose, 10 μM IPTG and different KCl concentrations under study. Microplates were incubated at 37 ºC with continuous orbital shaking.

Co-expression of Kup transporters and c-di-AMP

M9mod supplemented with 0,2 % Glycerol and 10 mM KCl was inoculated with -80 ºC stock strains under study and propagated twice. With the ON saturated cultures, 10 ml of fresh media were inoculated at OD = 0.05. When strains reached OD = 0.5, cultures were harvested and resuspended in same volume of M9mod supplemented with 0,2 % glycerol and no KCl. Samples were incubated at 37 ºC for an hour, after which 2 wash steps with no potassium supplementation were performed. These washed samples were used for microplate inoculation supplemented with 0,1 mM KCl and 2.5 μM IPTG. Genes cloned in pBAD33 vector were induced with 0.005 % L-arabinose when indicated. Growth curves were obtained as previously described.

Overproduction of proteins in *E. coli* BL21

Overexpression genes in *E. coli* BL21 was carried as follows. Overnight cultures of strains under study were diluted to an OD_{600} = 0.05 in 2 Lt. of fresh LB media supplemented with 100 μg/ml ampicillin. When an OD_{600} of 0.5 was reached, 0.5 mM IPTG was added to the cultures, which were further incubated for 3 h.

Overproduction of TScf in *L. lactis*

Expression of TScf was carried as follows. Overnight cultures of recombinant *L. lactis* were diluted to an OD_{600} = 0.05 in 3 Lt. of fresh M17G medium supplemented with the

corresponding antibiotics. Cultures were incubated at 30º C until an OD_{600} of 0.5 was reached, when 5 ng/ml of nisin (Sigma, USA) was added to the cultures. Samples were further incubated for 3 h.

Purification of His_{6X}-tagged proteins from *L. lactis*

After induction, *L. lactis* samples were collected by centrifugation and resuspended in lysis buffer. Cells were then lysed with a tissue lyser as previously described. The lysate was clarified by centrifugation, and NaH_2PO_4 and imidazole were added to a final concentration of 100 mM and 5 mM, respectively, pH was adjusted to 8.

Clarified lysates were applied to a Ni^{2+}-NTA affinity column (Qiagen) previously equilibrated with lysis buffer and incubated at room temperature for 1 hour to allow binding. Then the protein was refolded by successive in-column incubations with buffer C containing decreasing concentrations of urea ranging from 6 M to 0 M. The column was then washed with buffer C plus 25 mM imidazole and the protein was eluted from the column with buffer C with 500 mM imidazole. The purified protein was dialyzed against PBS plus 5 % glycerol; aliquots were kept at -80 ºC. Buffers are listed in Table XVIII.

Purification of His_{6X}-tagged proteins from *E. coli*

E. coli induced pellets from 2 Lt. cultures were washed in buffer W and cells were resuspended in buffer C, to be disrupted via French Press as previously described. Samples were then centrifuged 10 minutes at 5000 rpm and remaining insoluble particles were removed by ultracentrifugation at 4 ºC and 35000 rpm for 1 hour. For purification of recombinant His_{6X}-tagged proteins the supernatant fraction was loaded onto a bed of 1000 µl of Ni^{2+}-NTA resin (IBA) in a Poly-Prep Chromatography Column (BioRad) that had been pre-equilibrated in buffer C. After applying the cell extract, the column was washed 5 times with 5 ml of buffer C containing 10 mM imidazole. The elution was performed in steps of 5 ml, with increasing concentrations of imidazole (30 mM, 50 mM, 100 mM, 200 mM and 500 mM). Fractions were analyzed by SDS-PAGE and those were a band evidenced the presence of the protein of interest were pooled and concentrated by centrifugation in Vivaspin turbo 15 columns (Sartorius) for 15 minutes at 4.000 rpm and 8 °C.

Protein concentration was determined by Bradford assay and samples were further purified and analyzed *via* Size-exclusion chromatography (SEC). Buffers are listed in Table XVIII.

Size exclusion chromatography

The experiments were performed at room temperature in a HiLoad 16/600 Superdex 200 pg using an ÄKTAprime plus chromatography system (GE Healthcare Life Sciences). Purified protein was applied to the column previously equilibrated with 20 mM Tris-HCl, 50 mM NaCl, pH 8.0 buffer. The program was set to 1 ml/min and several elution fractions were recovered, where protein concentration was measured by a spectrophotometer coupled to the column. A calibration run was performed with molecular weight standards and related to their elution volumes for both column sizes. The elution volume is directly proportional to the $\log_{10}$(Mw).

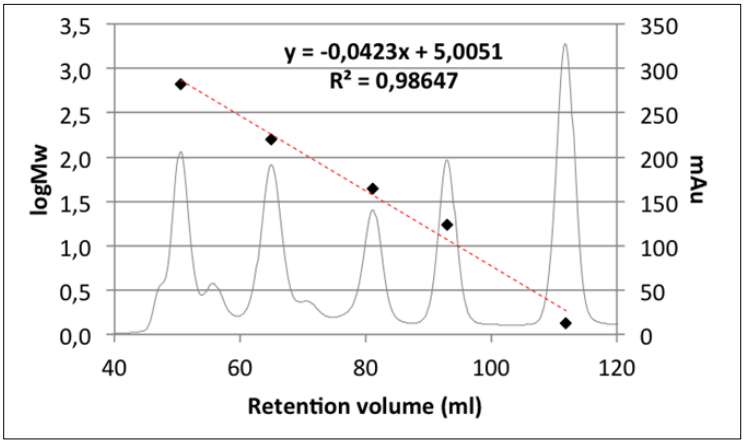

Fig 5 Standard curve in a HiLoad 16/600 Superdex 200 pg chromatography column. Proteins of different molecular weight were injected into the size-exclusion chromatography column. The grey line, corresponding to the secondary vertical axis represents the spectrum of elution. The logarithm of the molecular weight of each protein was plotted against its retention volume. The standard curve was then used to interpolate molecular weight from experimental retention times.

Table XI. Standard values for HiLoad 16/600 Superdex 200 pg

Mw (KDa)	$\log_{10}$(Mw)	Retention volume (ml)
670	2.83	50.45
158	2.20	64.92
44	1.64	81.19
17	1.23	93.02
1.35	0.13	111.92

2.3.9. Protein pull-down experiment with strep-tagged magnetic beads

Cultures of *L. lactis* IL1403 were grown in M17G medium until a final OD_{600} of 0.5, when cells were harvested at 10000 rpm for 5 minutes. Pellets were resuspended in 2 ml of CP1 buffer. This suspension was disrupted using French press and centrifuged 10 minutes at 10000 rpm and 4 ºC. The supernatant was split in two fractions of equal volume and centrifuged for 1 hour at 68000 rpm and 4 ºC. Protein quantification of the supernatant was then performed via Bradford assay.

1.2 mg of protein was then taken to a final volume of 1 ml CP1 buffer, with addition of glycerol 10 %, 0.004 % EDTA and 0.5 μg/ml BSA. This sample was then incubated for 0.5 hour at room temperature in a vortex shaker with the + and – beads (see below).

Strep-tactin beads were equilibrated with CP2 buffer. After equilibration, half of the tubes were incubated with biotinylated c-di-AMP in a vortex shaker for 15 minutes at room temperature (+ samples). Finally, the beads were washed twice with CP2.

After incubation with pre-equilibrated beads, samples were washed four times to get rid of unbound proteins and finally, elution was done with 50 μl buffer E at room temperature for 15 minutes in a vortex shaker.

Protein elution was analyzed by SDS-PAGE and silver staining. Both + and – lanes were sent for peptide identification at the proteomic department of the Ernst Moritz Arndt University, Greifswald (Prof. Uwe Völker). Buffers are listed in Table XVIII.

2.3.10. Differential radial capillary action of ligand assay (DrACALA)

Genes of interest were cloned in vector pWH844 using strain *E. coli* BL21 as host. An induction test was then performed as previously described to check gene expression (Zuzanna Grubek Master Thesis, 2016). Plasmids under study were finally sent to Prof. Vincent Lee (Department of Cell Biology and Molecular Genetics, University of Maryland, U.S.A) for the evaluation of specific interactions between their corresponding encoded proteins and c-di-AMP via the DrACALA assay.

2.3.11. Determination of c-di-AMP intracellular levels

20 ml cultures of *L. lactis* IL1403 were grown in M17G medium. When samples reached an OD_{600} of 0.50, cells were harvested at 4 ºC and 5000 rpm, and quickly frozen in liquid nitrogen. Two additional samples of 1 ml were taken for normalization purposes. Samples were collected and stored at -20 ºC until c-di-AMP extraction was performed. For this, pellets were resuspended in 150 µl 2 mg/ml lysozyme in TE buffer and incubated for 30 minutes at 25 ºC and 750 rpm. Afterwards, samples were frozen in liquid nitrogen and boiled at 95 ºC for 10 minutes. First, an extraction with 800 µl acetonitrile:methanol 1:1 was done, after which two more extractions with 200 µl acetonitrile:methanol:water 2:2:1 were performed. Supernatants were collected and dried in a Speedvac at 40º C. Pellets were sent to Prof. Volkhard Kaever from the Medizinische Hochschule, Hannover for c-di-AMP quantification via mass spectrometry. Final data was normalized with respect to the amount of protein present in the sample, determined via Bradford assay.

2.3.12. Bis(p-nitrophenyl)phosphate assay.

Bis(p-nitrophenyl)phosphate (BNPP) assay was performed according to Bai et al. 2013 (68). Briefly, the following reaction is set up in a final volume of 100 µl:

Putative phosphodiesterase	0.1 µM
Tris-HCl pH 7.5	100 mM
Mn_2Cl	100 µM
NaCl	10 mM
BNPP	2 mM
$_dH_2O$	for V_f = 100 µl

Reactions were loaded in microplates and were followed in a microplate reader at 30 ºC and λ = 410 nm during 4 hours. Incubation was performed with minimal reading intervals and medium shake.

2.3.13. Light microscopy

For microscopic analysis of *L. lactis* samples, cells were grown in M17G medium at 30º C to the desired OD_{600}. A volume of 0.3 µl was placed on a slide covered with a thin layer of 1% agarose prepared in $_dH_2O$, and when dried, a coverslip was placed on the sample. Pictures were taken using the AxioImager M2 equipped with a digital camera AxioCam MRm and the AxioVision Rel 4.8 software for image uptake (Zeiss). The objectives used ECPlan-NEOFLUAR 100X/1.3 (Zeiss).

2.3.14. Survival analysis in *Galleria mellonella*

Survival experiments in *G. mellonella* were performed as follows: larvae were selected according to their weight, which was between 0.18 and 0.35 grams. 32 individuals per group were then harvested ON at 30 °C. Each group was inoculated with bacterial suspensions made in PBS at a concentration of 9.10^6 CFU/larva. Inoculation site was the fifth proleg.

After inoculation, larvae were monitored at 2-4 hours intervals for 72 hours. Then, data was plotted in Kaplan-Meier curves using SigmaPlot 12 software and employing LogRank and Holm-Sidak tests, for multiple comparisons. P value was set in 0.05 (151).

2.3.15. Immunological experiments

Preparation of live bacterial inoculum and immunization protocol

2 Lt. cultures of M17G supplemented with the corresponding antibiotics and 15 ng/ml of nisin were inoculated with *L. lactis* strains of interest at $OD_i = 0.05$. Samples were incubated at 30 ºC until OD_{600} reached 0.5. Cells were then centrifuged at 5000 rpm and 4 ºC for 10 minutes and washed with PBS buffer, to be finally resuspended in PBS plus 10 % glycerol. CFU were determined in the samples to check concentrations of the range $2 – 4.10^9$ CFU/ 100 µl inoculum. In addition, an expression test was performed to corroborate the presence of TScf.

Mice inoculation

BALB/c female mice, aged 6 weeks, were acquired and housed in HEPA-ventilated racks at 21 – 22 ºC and 68 % humidity at the animal facility of the CIPREB (Center for Research and Production of Biological Reagents, School of Medicine, UNR). Animals had free access to

food and water and were maintained under a 12 hours light/dark period. All protocols for animal studies were performed in collaboration with Dra. Ana Rosa Pérez and Dr. Iván Marcipar groups from the Instituto de Investigación Clínica y Experimental de Rosario (IDICER CONICET-UNR) and the Laboratorio de Tecnología Inmunológica, Facultad de Bioquímica y Ciencias Biológicas, UNL (Universidad Nacional del Litoral), respectively. Moreover, these experiments were approved by the Bioethics and Animal Care & Use Committees according to Institutional guidelines.

Briefly, mice were immunized by oral route by three successive doses separated by two-week intervals by gavage administration. The bacterial dose administered was set as a quantity of bacteria expressing 10 µg of TScf (0.3-1.10^6 CFU). Similar quantities of bacteria expressing the TScf antigen alone, the CdaA alone or the empty vector were administrated in parallel. For comparative purposes, a group of animals was subcutaneously immunized in parallel with 10 µg of purified TScf adjuvanted with 3 µg of ISPA ("*Gold standard*" group), an ISCOMATRIX type adjuvant developed by Dr. Marcipar group (152).

Cellular response (Delayed hypersensibility test)

To test cellular response, mice were challenged with 5 µg of purified TScf by intradermal injection in the right footpads 12 days after the last immunization. The thickness of hind footpads was measured before and 24, 48 and 72 hours after the antigen injection with a digital Vernier caliper. Results of the delayed hypersensitivity test (DHT) were expressed as the difference in thickness of footpads after and before the inoculation.

3. Results

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we fear less" Marie Curie

3.1. c-di-AMP and potassium uptake in *L. lactis* IL1403

3.1.1. Identification of c-di-AMP interaction partners in *L. lactis* IL1403

In an attempt to identify new interaction partners of c-di-AMP in *L. lactis* IL1403, a protein pull-down experiment was performed (50). As described in Materials and Methods, cell extracts of this strain were incubated with strep-tagged magnetic beads previously coupled to biotinilated c-di-AMP (+ sample). A sample incubated with the beads without previous treatment was used as negative control (- sample). Elutions were analyzed by SDS-PAGE and silver staining (Fig. **6**, I) and both lanes were sent for peptide identification by mass spectrometry. A list of 151 different proteins was obtained, with the ratio of their presence in the + lane with respect to the – lane. For the list of peptides obtained after the pull-down experiment, see Table XIV in the Appendix.

It is important to mention that biotinilated proteins would also bind to the strep-tag in the – sample, which is probably why pyruvate carboxylase was identified almost 50 times more represented in the – lane. On the other hand, secondary interaction partners, i.e. proteins interacting with c-di-AMP binding proteins, could also be eluted depending on the strength of the interaction. Moreover, if the interaction to c-di-AMP is not strong enough, some binding partners could also be washed out, or found with a low +/- ratio. To evaluate all these possibilities, a bibliographic search was carried out, although the vast majority of the proteins on the list are either uncharacterized, or no studies about them have so far been reported. Since there is no information available about which proteins are biotinilated, nor which are essential in *L. lactis* (which could lead to the essential role of c-di-AMP in this bacterium), an initial classification was made by use of the UniProt online resource.

Fig. **6**, II shows a functional classification of the 130 peptides with the highest +/- ratio, based on available information and amino acid sequence homology to previously studied proteins from other bacteria. As the pie chart shows, the most represented groups include transport proteins comprising a predicted glutamine ABC transporter, a glycine-betaine transporter, four putative ABC transporters and two uncharacterized proteins. Also, a considerable amount of proteins related to DNA metabolism were identified, such as the DNA polymerase III, subunits β and τ, the DNA mismatch repair proteins MutS and MutL, and the predicted proteins RecG (DNA helicase), RecN (DNA repair) and RecA (DNA

repair/homologous recombination). Finally, groups of proteins related to amino acid and cell wall metabolism were also among the most represented, including predicted amino acid synthesizing enzymes and proteins involved in the formation of the septal ring and cell division (EzrA, FtsA and FtsE, which was proved to affect translocation of K^+ in *E. coli* (37)).

Considering that actually most of the proteins showed a great variety of predicted functions and therefore they were collected under "Miscellaneous proteins", no clear tendency could be distinguished. Altogether, for the subsequent specific interaction assay, the 23 peptides with the highest +/- ratio were selected. In other words, this selection established a cut off of proteins which were at least eight times more represented in the + lane than in the – lane (Table XII). Due to the proximity of the ratio value to the set cut off, the uncharacterized protein with an accession number Q9CEI1_LACLA, and the predicted septation ring formation regulator EzrA were as well added to the list.

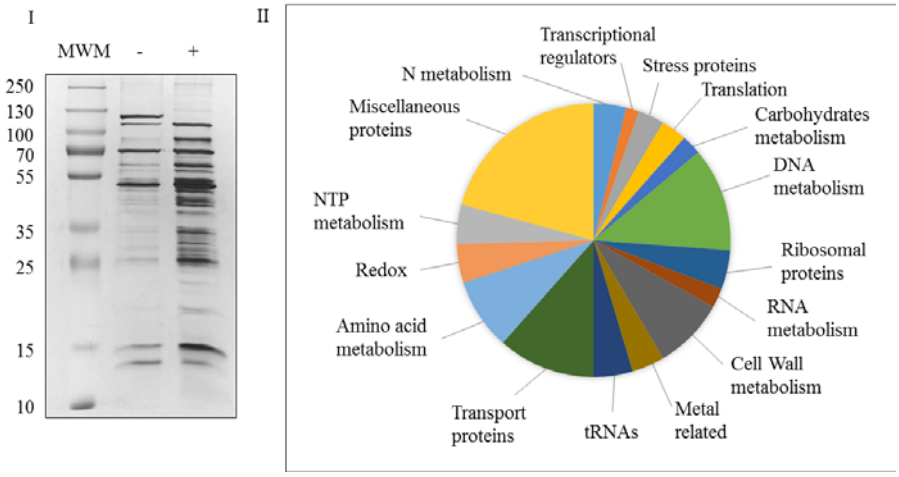

Fig. 6 Protein pull-down for identification of c-di-AMP interaction partners. I. SDS-PAGE after pull-down experiment with strep-tagged magnetic beads (see Materials and Methods). Bands were visualized by silver staining. + lane: sample eluted from beads previously treated with biotinilated c-di-AMP. – lane: sample eluted from beads with no previous treatment. MWM: Molecular weight marker (PageRulerTM Plus Prestained Protein Ladder). This experiment was performed in three independent replicates. Both lanes were sent for peptide identification. II. Functional classification of proteins obtained after MS/MS detection. Protein function was assigned based on reported research and sequence homology to previously studied proteins.

Furthermore, at the time this experiment was performed, an increasing amount of evidence was directing the essential role of *cdaA* (and consequently c-di-AMP) towards its relation to potassium metabolism (153). Consequently, a search for genes coding for putative potassium transporters, and proteins potentially related to potassium homeostasis in *L. lactis* IL1403 was carried out.

When looking for potassium transporters encoded in *L. lactis* IL1403 genome by use of the free online software UniProt (UniProtKB, UniRef and UniParc databases), proteins KupA, KupB and YrbD are shown as results. These three proteins are inferred from homology since there is currently no reported research about them. The first two belong to the KT/KUP/HAK family (see below), whereas YrbD could be a voltage gated channel. Since neither potassium metabolism, nor its relation to c-di-AMP has been thoroughly investigated in *L. lactis*, the possibility of this second messenger interacting as well with potassium channels was considered. Thus, these proteins were added to the list along with the putative voltage gated potassium channel proteins YjdJ and YncB, with more than 60% identity to the potassium efflux system KefA from other *Lactococcus* species.

Proteins with high homology for members of the Trk/Ktr/HKT family were also evaluated. Interestingly, even though putative homologous proteins to *B. subtilis* KtrA were found in some members of *L. lactis* species, it is not present in IL1403 nor in MG1363 strains studied in this work. Moreover, even though the *kim* riboswitch was already reported to be absent in *Lactococcus* (154), the presence of the associated potassium transporter KimA, or possible related proteins was also considered. No KimA homologues were found encoded in *L. lactis* genome, but the protein blast showed certain homology to an amino acid permease, LysP. Given the recent associations of c-di-AMP to amino acids metabolism and osmoregulation previously described in the introduction (62, 155), both LysP and LysQ were also included in the list of putative c-di-AMP interaction partners.

It was also reported that in *L. monocytogenes* a mutant strain for the only c-di-AMP synthesizing enzyme was obtained in rich media thanks to loss of function mutations occurring in genes clusters *oppABCDF* (involved in oligopeptide import) or the *gbuABC* (involved in glycine-betain import) (60). Therefore, the homologous Opp protein of *L. lactis* IL1403, which is expected to function as an oligopeptide permease, was added as well to the list.

Lastly, and due to the close connection between potassium and sodium homeostasis, Nah protein, homologous to a cation:proton antiporter was also selected. In conclusion, a final selection of 34 genes (Table XII) was then cloned in the *E. coli* expression vector pWH844 (Zuzanna Grubek Master Thesis, 2016). After checking expression of all genes using strain *E. coli* BL21 as host, the corresponding plasmids were sent to confirm c-di-AMP specific interaction to the respectively encoded proteins via differential radial capillary action of ligand assay (DrACALA) (see Materials and Methods).

Table XII. Gene selection for DrACALA analysis

Gene	Putative function	Reference
dnaH	DNA polymerase III, subunits β and τ[1]	Bolotin et al. 2011
ezrA	Septation ring formation regulator[1]	Bolotin et al. 2011
ftsE	Control of cell wall synthesis; ABC transporter[1]	Bolotin et al. 2011
glnP	Glutamine ABC transporter permease and substrate binding protein[2]	Fulyani et al. 2013 (156)
kupA*	Potassium transporter[2]	This work
kupB*	Potassium transporter[2]	This work
lysP*	Lysine specific permease[1]	Bolotin et al. 2011
lysQ*	Lysine specific permease[1]	Bolotin et al. 2011
mvaA	3-hydroxy-3-methylglutaryl coenzyme A reductase[1]	Bolotin et al. 2011
nah*	Na^+/H^+ antiporter[1]	Bolotin et al. 2011
oppA*	Oligopeptide-binding protein[1]	Bolotin et al. 2011
pdp	Pyrimidine-nucleoside phosphorylase[1]	Bolotin et al. 2011
prfB	Peptide chain release factor 2[1]	Bolotin et al. 2011
racD	Aspartate racemase[1]	Bolotin et al. 2011
recG	ATP-dependent DNA helicase[1]	Bolotin et al. 2011
recJ	Single-stranded DNA specific exonuclease[1]	Bolotin et al. 2011
recN	DNA repair protein RecN[1]	Bolotin et al. 2011
relA	ppGpp synthetase I[1]	Bolotin et al. 2011
ribH	6,7-dimethyl-8-ribityllumazine synthase[1]	Bolotin et al. 2011
rpsF	30S ribosomal protein S6[1]	Bolotin et al. 2011
yciH	Ribonuclease J[1]	Bolotin et al. 2011
yeaD	Initiation-control protein YabA[1]	Bolotin et al. 2011
yhfC	Uncharacterized protein	Bolotin et al. 2011
yjdJ*	Potassium channel protein[1]	Bolotin et al. 2011
yncB*	Uncharacterized protein	Bolotin et al. 2011
yqaB	Uncharacterized protein	Bolotin et al. 2011
yqaD	Uncharacterized protein	Bolotin et al. 2011
yrbD*	Uncharacterized protein	Bolotin et al. 2011
yrgI	Uncharacterized protein	Bolotin et al. 2011
yrjB	Oxidoreductase[1]	Bolotin et al. 2011
ytcE	Uncharacterized protein	Bolotin et al. 2011
ytjA	Uncharacterized protein	Bolotin et al. 2011
yueF	Protease[1]	Bolotin et al. 2011

ywfH	Uncharacterized protein	Bolotin et al. 2011

[1]Proteins inferred from homology. [2]Experimental evidence available. *Genes selected independently from the pull-down experiment

3.1.2. c-di-AMP specific interaction to different Lactococcal proteins

Unexpectedly, the majority of the genes selected proved to have no specific interaction with c-di-AMP through the DrACALA assay. Nevertheless, two of the putative K^+ related proteins threw positive results. The corresponding genes were *kupA* and *kupB* and their products interact specifically with c-di-AMP according to the competition DrACALA experiment (Fig. 7). In addition, the interaction with KupA seems to be stronger than with KupB.

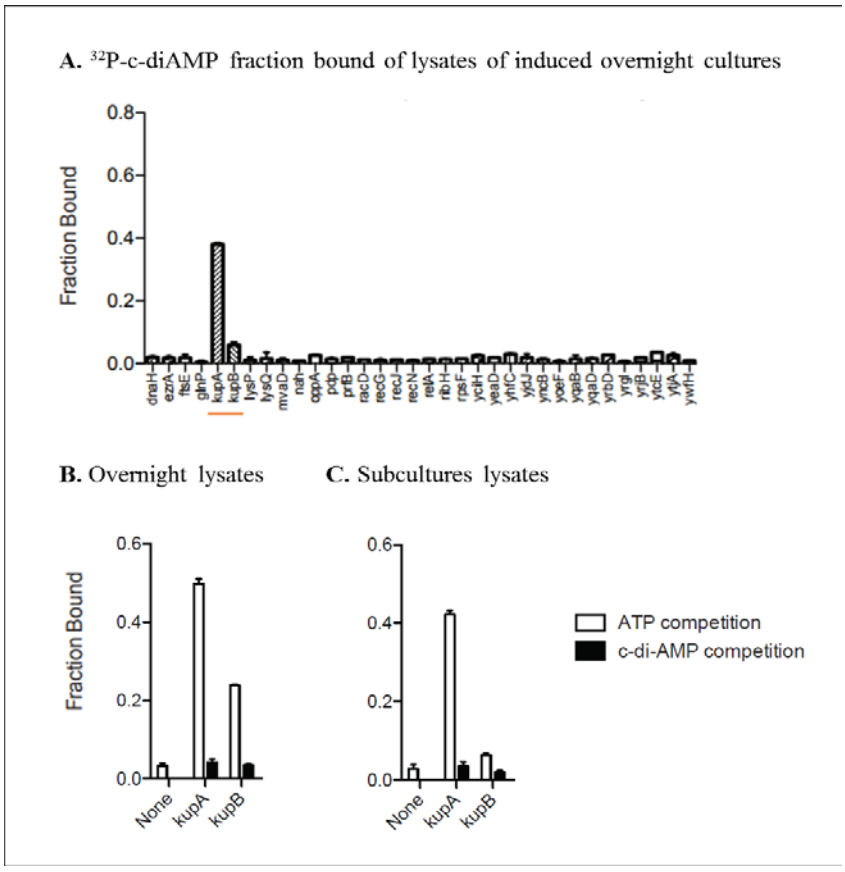

Fig. 7 DrACALA experiment. A: Fraction of radiolabeled c-di-AMP retained in induced overnight cultures. KupA and KupB specifically interact to c-di-AMP (underlined in orange). ATP competition does not interfere with c-di-AMP interaction in overnight (B) or subcultures lysates (C). Fresh c-di-AMP competition results in no ^{32}P-c-di-AMP detected, indicating specific interaction. Induction of all samples was checked in overnight and midlog cultures (not shown).

Because of the lack of previously reported potassium transporters in *L. lactis* IL1403, a bioinformatic search was first performed. An initial approach showed that Kup proteins are

widely found in bacteria as well as in species of fungi and plants. Nevertheless, the only reported investigation related to a Kup protein in *L. lactis* dates from 1997 when Quintero and Blatt analyzed a new transporters family in *Arabidopsis* and they see that it is conserved among different filos, since homologous sequences were found in different plant species, in *L. lactis* and also in *Homo sapiens* (157). However, up to date no other information can be found, which is why an *in silico* analysis of KupA and KupB was carried out.

In the model organism *E. coli*, Kup acts as a potassium intake system with particular characteristics that distinguish it from the potassium transporter families Ktr and Kdp, previously described. It belongs to the Kup/HAK/KT family and, as Fig. 8 represents, it is a 622 residues protein with 12 transmembrane fragments at the N-terminal end (158).

A first study of the nucleotidic sequence of *L. lactis* IL1403 genome shows two adjacent genes of 2013 and 2016 base pairs, corresponding to *kupA* and *kupB* respectively, separated by 223 bp (Fig. **8**, I). The amino acid sequence alignment between them shows a 73% identity, whereas topology prediction using the free software Protter threw very similar structures with 12 transmembrane segments for both proteins under study, which correlates with *E. coli* Kup protein. Moreover, as Fig. 8 II shows, the majority of conserved residues are located in the N-terminus and particularly in the transmembrane regions. This makes sense provided *E. coli* does not synthesize c-di-AMP and, consequently, its Kup protein does not need to sense it. It is hence likely that in *L. lactis* the sensory domain has evolved from a common ancestor in the cytoplasmic C-terminal region, to acquire the capacity of c-di-AMP sensing and responding accordingly, modifying K^+ intake activity.

On the other hand, and even though the presence of acidic amino acids has been shown to be important for potassium transport, in Kup they are found in the transmembrane fragments of the protein. This has been reported as a unique feature of the Kup/HAK/KT family, not occurring neither for transporters of the Trk/Ktr/HKT nor for the Kdp family (158). Moreover, it was also proved that *E. coli* Kup residues D23, E116, E229 and D408 have a crucial role for Kup activity and its substitution for alanines resulted in a peptide that could not complement mutations in *E. coli* LB2003 to allow growth at low K^+ concentrations (see below). Interestingly, D23 and E229 are conserved in both Kup proteins of *L. lactis* IL1403 and despite E116 and D408 are not, in both KupA and KupB an aspartic acid and a glutamic acid residue are found instead respectively, so conservation of acidic residues remains (Fig. 8).

On the other hand, sequence alignment of well-studied c-di-AMP binding potassium transporters shows a conserved glycine region, characteristic of Ktr family proteins. This GXGXXG consensus motif, typically present for nucleotide binding is not exactly conserved in Kup proteins but a GXXGXG inverted domain is found instead in both KupA and KupB (Fig. 8, II). This domain could be involved in c-di-AMP binding or could also suggest that Kup transporters need a cofactor like NAD for their activity (159).

Taken together, the evidence here presented supports the fact that proteins under study belong to the Kup/HAK/KT family but whether they are both active potassium transporters needed to be further studied.

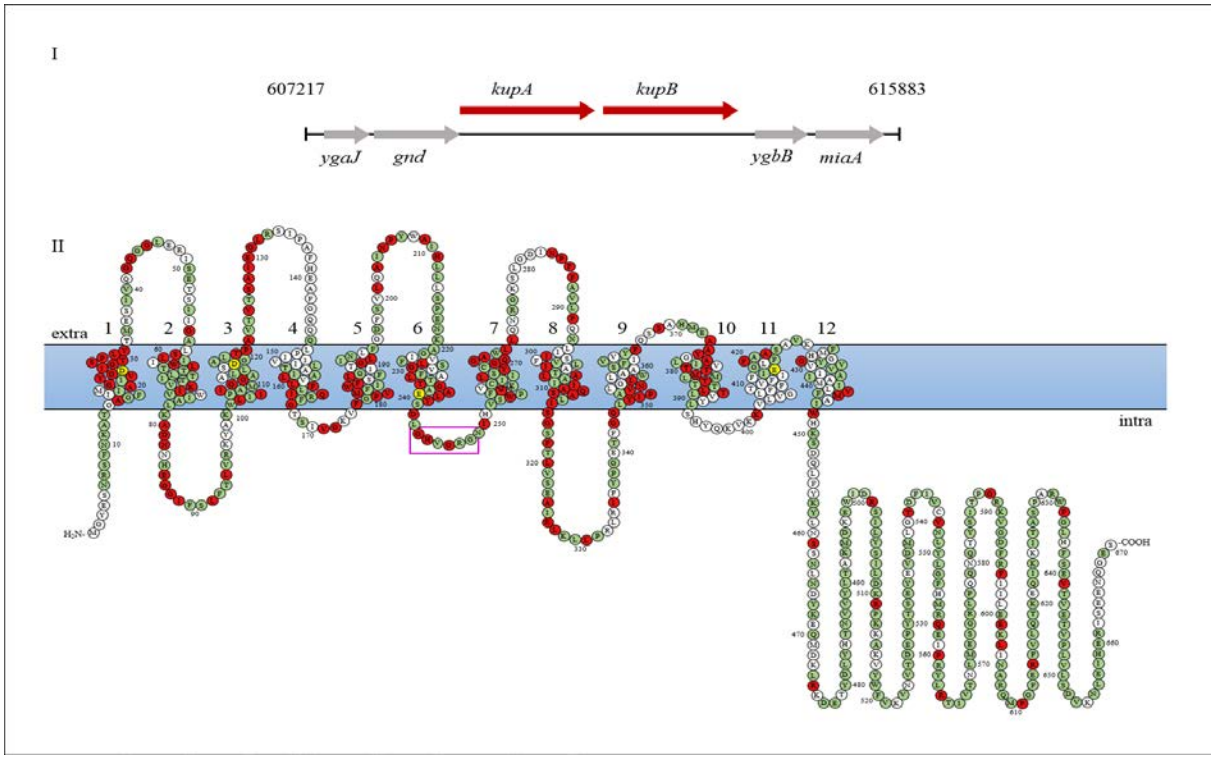

Fig. 8 KupA and KupB *in silico* analysis. I: Gene arrangement in *L. lactis* IL1403 genome. *kupA* and *kupB* are 2013 and 2016 base pairs genes respectively and are adjacently encoded. II: Predicted membrane conformation of KupA. Adapted from the free topology prediction Protter software. Amino acids conserved in both Kup proteins from *L. lactis* are highlighted in green, whereas those conserved in *E. coli* as well are represented in red. Conserved acidic amino acids are depicted in yellow. The cellular membrane is shown in light blue. Extra: extracellular, intra: intracellular. The purple box indicate a putative GXXGXG c–di-AMP binding site.

3.1.3. KupA and KupB of *L. lactis* 1403 restore growth of *E. coli* LB650

Heterologous expression of proteins in the model bacterium *E. coli* has always been one of the first choices when studying unknown protein functions. Consequently, a first approach into the functional properties of the proteins encoded by both *kup* genes was explored using *E.*

coli LB650 (160). This strain is a triple mutant for the main potassium transporter systems, having a Δ*kdpABC5* Δ*trkH* Δ*trkG* genotype (see Table XV). Hence, it is unable to grow at low K^+ concentrations.

kupA and *kupB* genes were respectively cloned in a modified version of plasmid pWH844, lacking the N-terminal His_{6X}-tag after digestion with EcoRI restriction enzyme, which site is located upstream the His_{6X} -tag codifying sequence. For this, primers IQ682 and IQ683 were used, which introduce an EcoRI restriction site as well as ribosome binding site. In this way, any functional interference produced by the histidine residues in the proteins was ruled out. The resulting plasmids pIQ309 and pIQ310, (carrying *kupA* and *kupB* genes, see Table XVI), were electroporated in *E. coli* LB650, originating strains LB09 and LB10 respectively, to check if their expression could restore growth in rich media when no KCl is added. As Fig. 9 shows, the strain harboring pWH844 vector cannot grow in LB, unless it is supplemented with 200 mM KCl. On the other hand, when a *trans* copy of *kupA* or *kupB* is present, colonies appear on the plates without extra addition of KCl, even when IPTG is not present. As mentioned in Material and Methods, pWH844 vector used for these constructions has the multiple cloning site under control of the lactose operon promoter (Plac), which is widely known to be leaky. Consequently, this basal expression of *kupA* and *kupB* is already enough to allow growth. These results show not only that the proteins under study are potassium transporters but also suggest that both transporters have high affinity for K^+.

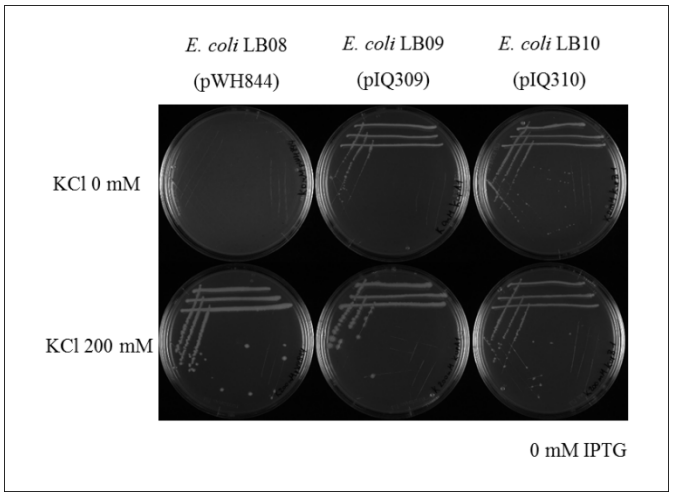

Fig. 9 KupA and KupB restore growth of *E. coli* LB650 in LB. Left: strain LB08 carrying pWH884 vector grows only when 200 mM KCl is added to the medium (down). Center and right strains LB09 and LB10 carrying a *trans* copy of *kupA* and *kupB*, respectively. Both evidence growth with or without potassium supplementation.

3.1.4. KupA and KupB are high affinity transporters

In order to estimate the affinity of KupA and KupB proteins for K^+, a comparison to previously characterized transporters was made. For this, the high affinity potassium transporter KtrA/B from *B. subtilis* and the low affinity KtrC/D system from *L. monocytogenes* were employed (48, 161). The corresponding genes had been previously cloned in the modified version of pWH844 plasmid described in the former section, lacking the His_{6X}-tag. The resulting plasmids pBP372 and pBP371 were available at Prof. Stülke's lab and were electroporated in strain *E. coli* LB650, originating strains LB11 and LB12, respectively.

Growth curves were then performed in minimal salt media M9 modified, where potassium salts were replaced by equimolar quantities of the respective sodium salt (M9mod, see Materials and Methods). KCl was then added to the media, to reach final concentrations between 0.025 – 50.0 mM, and determine in this way the concentration at which growth was possible. OD_{max} and μ_{max} were then determined for strains *E. coli* LB08, LB09 and LB10, harboring the empty vector, and *kupA* and *kupB* genes, respectively. Strains LB11 and LB12 were included as reference of the response of a high and a low affinity system under the conditions here studied.

Both Kup proteins showed similar phenotypes, manifesting growth even at concentrations below 0.1 mM KCl, although growth rate and final OD_{600} were low (Fig. 10 and Table XIII) Moreover, at 1.0 mM KCl, values close to the highest OD_{max} and μ_{max} are already reached, and coincide with values obtained for the high affinity system KtrA/B of *B. subtilis*. On the other hand, for the case of KtrC/D, growth is only possible at 10 mM K^+ or higher, and maximum OD_{max} and μ_{max} values are obtained when at least 50 mM K^+ is present in the media. A marked difference is then established with respect to high affinity KtrA/B and Kup proteins, being more closely related to strain LB08 harboring the empty vector. Taken together, these results support the theory that both Kup proteins are high affinity potassium transporters.

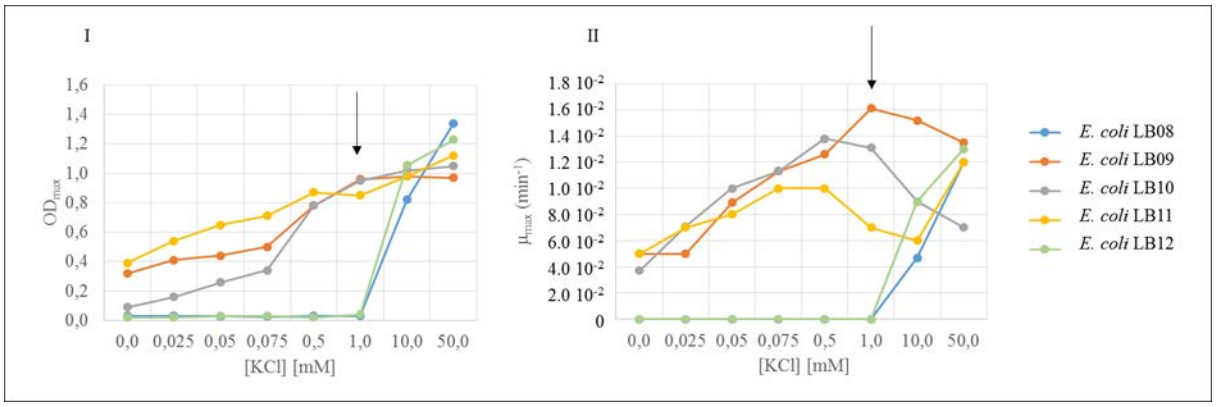

Fig. 10 Growth parameters of *E. coli* LB650 derived strains. Growth curves were performed in a microplate reader and in minimal salt M9mod medium. OD_{max} (I) and μ_{max} (II) were determined at different KCl concentrations. The arrow highlights parameters obtained at 1.0 mM KCl, where the different potassium transporter systems show clear distinct phenotypes. Both Kup proteins behaved similarly to the high affinity KtrA/B system.

Table XIII. OD_{max} and μ_{max} values for *E. coli* LB650 derivative strains

	pWH844		KupA		KupB		KtrA/B		KtrC/D	
$[K^+]$ [mM]	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}
-	ND	ND	0.3	5.0E-3	0.1	3.7E-3	0.4	5.0E-3	ND	ND
0.025	ND	ND	0.4	5.0E-3	0.2	7.1E-3	0.5	7.0E-3	ND	ND
0.50	ND	ND	0.4	8.9E-3	0.3	1.0E-2	0.7	8.0E-3	ND	ND
0.075	ND	ND	0.5	1.1E-2	0.3	1.1E-2	0.7	1.0E-2	ND	ND
0.50	ND	ND	0.8	1.3E-2	0.8	1.4E-2	0.9	1.0E-2	ND	ND
1.0	ND	ND	1.0	1.6E-2	1.0	1.3E-2	0.9	7.0E-3	ND	ND
10.0	0.8	4.7E-3	1.0	1.5E-3	1.0	9.0E-3	1.0	6.0E-3	1.1	9.0E-3
50.0	1.3	1.2E-2	1.0	1.6E-2	1.1	7.0E-3	1.1	1.2E-2	1.2	1.3E-2

3.1.5. c-di-AMP down-regulates Kup proteins

Once specific interaction with c-di-AMP was confirmed, and KupA and KupB were identified as potassium transporters, the next step was to analyze the impact of this metabolite on the activity of both Kup proteins. To this aim, a co-expression system was established in the model bacterium *E. coli* 2003. This strain is deficient in the three major potassium uptake systems Trk, Kup and Kdp *(Δkdp*ABC5 *kupD1* Δ*trkA*, see Table XV), and is not able to grow in minimal salt media at low K^+ concentrations without a *trans* complementation of a potassium transporter coding gene. Very importantly, and in contrast to strain LB650 (Km^{50} – Cm^{30}), its mutations are clean, so there is no need for antibiotic supplementation of growth media. Moreover, *E. coli* lacks c-di-AMP synthesizing enzymes, for which the co-expression of *cdaA* and *kup* genes allows the analysis of the phenotypic effect of c-di-AMP on Kup proteins, without interference of host-synthesized c-di-AMP.

At the beginning of this thesis, several attempts were made to heterologously produce CdaA enzyme from *L. lactis* IL1403 in *E. coli*. Since no successful results were obtained, and taking into account that for the practical scope of the experiment only the production of c-di-AMP was necessary, independently of the synthesizing enzyme, CdaA from *L. monocytogenes* (from now on $CdaA^{lmo}$) was used instead. A non-active defective version of it, $CdaA^{lmo*}$, was used as well, as negative control.

Plasmids pIQ309 and pIQ310 (Amp^{100}) were used for *kup* genes induction via IPTG, whereas $cdaA^{lmo}$ and $cdaA^{lmo*}$ were induced from plasmids pBP370 and pBP373 respectively (kindly supplied by Prof. Stülke laboratory). These plasmids are pBAD33 derivatives (Cm^{30}, see Table XVI), allowing expression of genes under control of the arabinose Para promoter, and being therefore compatible with pWH844 derivatives previously mentioned. A system of co-expression was then established where potassium transporters could be induced by IPTG and c-di-AMP cyclases by arabinose.

In the previous section, where growth experiments were performed in *E. coli* LB650 harboring a *trans* copy of *kupA* and *kupB*, addition of 0.1 mM KCl to M9mod medium was enough for the strains under study to grow. Hence, this concentration was selected to supplement M9mod medium for the growth curves performed on the *E. coli* 2003 derivative strains. It is important to mention that under this potassium concentration, strain *E. coli* 0380, harboring vectors pWH844 and pBP370 ($cdaA^{lmo}$), is not able to grow, which is why it was used as negative control.

As seen in Fig 11, IPTG induction of *kupA* gene allowed growth of strains *E. coli* 0390 and 0393. Nevertheless, when $cdaA^{lmo}$ (present in strain 0390) is also induced via arabinose supplementation in growth media, a severe growth detriment is evidenced (Fig 11, left). On the other hand, either when the inductor (arabinose) is not present (Fig 11, right), or when *cdaA* gene carries a mutation that results in an inactive enzyme (strain 0393), growth is possible again.

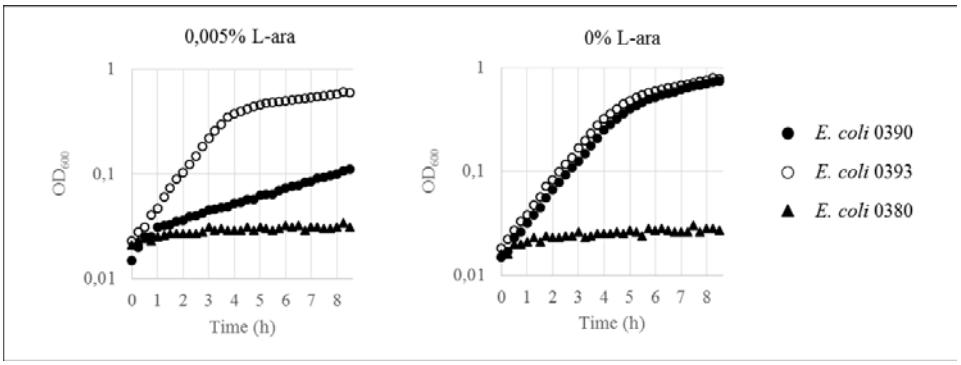

Fig 11 c-di-AMP effect on KupA. Growth curves were performed in minimal salt M9mod media, supplemented with 2.5 μM IPTG for *kupA* induction and with 0.005% L-arabinose (left) or without it (right). Strain 0390 and 0393 harbor a copy of *cdaA*^{lmo} and *cdaA*^{lmo*}, respectively. Both carry as well a copy of *kupA*. *E. coli* 0380 with the empty vector pWH844 and pBP370 was used as negative control.

The same experiment was performed to study the impact of c-di-AMP on KupB. In this case, results obtained were similar, but the growth inhibition exerted on strain 0300 (harboring plasmids pIQ310 with a copy of *kupB* and pBP370, with wild type *cdaA*^{lmo}) in induced conditions, was milder (Fig 12, left).

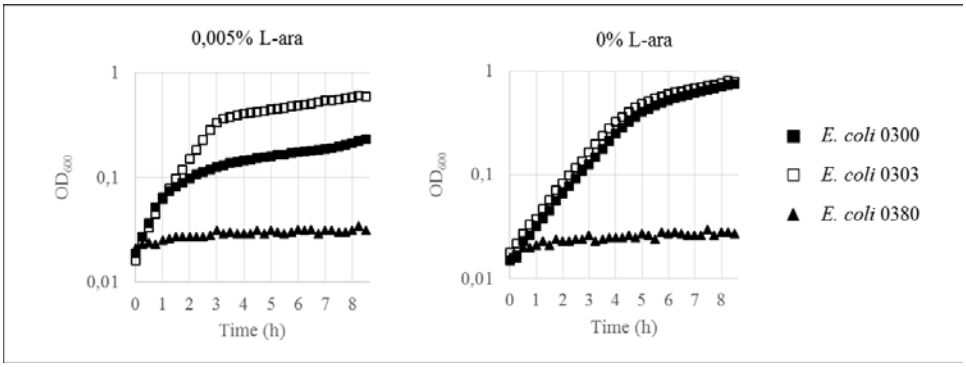

Fig 12 c-di-AMP effect on KupB Growth curves were performed in minimal salt M9mod media, supplemented with 2.5 μM IPTG for kupB induction and with 0.005% L-arabinose (left) or without it (right). Strain 0300 and 0303 harbor a copy *cdaA*^{lmo} and *cdaA*^{lmo*}, respectively. Both carry as well a copy of kupB. *E. coli* 0380 with the empty vectors pWH844 and pBP370 was used as negative control.

It is important to mention that the growth inhibition observed in both cases could be caused by high intracellular c-di-AMP concentrations, which have been reported to be toxic for bacteria (54). On the other hand, it could also be due to the production of the heterologous CdaA^{lmo} enzyme, which could be toxic for the cell independently of c-di-AMP concentration or the presence of the potassium transporters under study. This was ruled out by performing similar growth curves of *E. coli* 0380 harboring an empty copy of vector pWH844 and plasmid pBP370. This time, 50 mM KCl was added to growth media, and wild type growth was possible

under arabinose induction as well as no-induction conditions (Fig 13). As expected, strain 0383 harboring the mutated *cdaA*^{lmo*}, was not inhibited either and both strains evidenced similar growth phenotypes to strain 4433, with both empty vectors pWH844 and pBAD33. Altogether, the evidence here presented confirms that c-di-AMP downregulates KupA and KupB proteins.

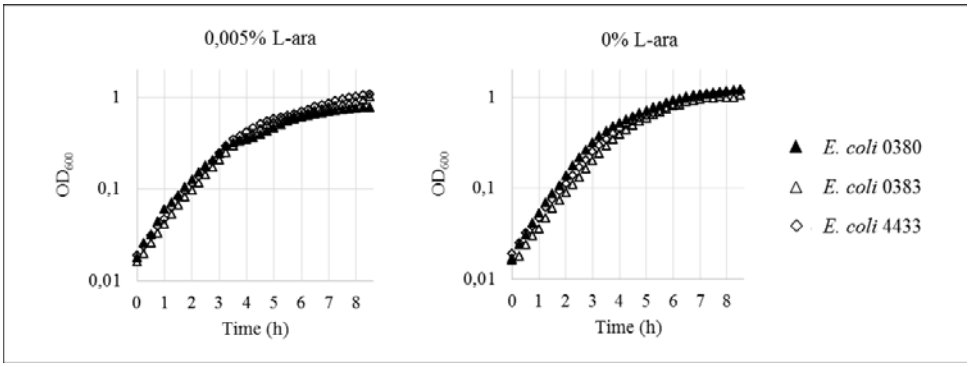

Fig 13 Control curves on M9mod supplemented with 50 mM KCl. High potassium concentrations allowed similar growth under arabinose induction (left) and no-induction conditions (right). 0380: pWH844 plus pBP370 (cdaAlmo), 0383: pWH844 plus pBP373 (cdaAlmo*), 4433: pWH844 plus pBAD33.

3.1.6. Conclusions

In this chapter, a pull-down experiment with strep-tagged magnetic beads coupled to biotinilated c-di-AMP was performed in order to identify novel interaction partners of this second messenger in *L. lactis* IL1403. Even though no specific binging protein was recovered from this experiment, the DrACALA assay permitted the identification of two novel potassium transporters, which specifically interact with c-di-AMP: KupA and KupB. Minimal KCl concentrations restoring growth of mutant strains *E. coli* LB650 and 2003 in M9mod medium, as well as comparisons to previously studied high and low affinity systems (*B. subtilis* KtrA/B and *L. monocytogenes* KtrC/D, respectively) provide evidence that, unlike in *E. coli*, *L. lactis* Kup proteins are high affinity potassium transporters.

An *in silico* analysis showed that KupA and KupB share 73% identity and when compared to *E. coli* Kup, the most conserved regions are in the transmembrane fragments, whereas the major variability occurs in the cytosolic C-terminal end. Moreover, after sequence analysis of other c-di-AMP binding proteins, a putative c-di-AMP binding domain was hypothesized.

Finally, a co-expression system was constructed, allowing controlled induction of *kup* genes under study and *cdaA*, by use of an IPTG and an arabinose inducible promoter,

respectively. Growth curves performed under low potassium concentrations confirmed that c-di-AMP has an inhibitory effect on both KupA and KupB.

3.2. Studies on c-di-AMP synthesizing and degrading enzymes in *L. lactis* IL1403

3.2.1. Strain engineering for modification of intracellular c-di-AMP levels

The first approach aiming to modify intracellular levels of c-di-AMP in *L. lactis* was directed towards deletion of the gene encoding its only synthesizing enzyme, *cdaA*. Several attempts were made by use of vector pBVGh, previously developed in our laboratory and described below. Since deletion in rich medium M17G was not possible, and mutants for c-di-AMP cyclases were reported on strictly controlled minimal media, the deletion protocol was tuned to be performed in defined media. As previously described, lactic acid bacteria lack many anabolic pathways and, consequently, a considerable amount of nutrients have to be provided in growth media, in order to satisfy their complex metabolic requirements. Different conditions were thus tried, to establish a suitable defined medium, which allowed regulation of potassium concentration and, at the same time, high *L. lactis* cellular density.

Minimal medium MS15 was employed as described by Cocaign-Bousquet et al. (162) and using the same criteria as for M9 minimal salt medium in the previous chapter, potassium salts were replaced by equimolar quantities of the corresponding sodium salt, resulting in MS15m medium. Different KCl concentrations were then used to supplement MS15m and determine the minimum potassium molarity allowing an OD_{600} high enough to perform the different steps of the deletion protocol. Fig 14, I shows the final OD_{600} obtained for *L. lactis* IL1403 and NZ9000 strains after 5 days of incubation at 30º, when 1.0 – 5.0 – 10 – 100 and 200 mM KCl were added to MS15m. Since the original protocol requires several ON incubation steps, use of this medium would be practically unviable.

On the other hand, Zhang et al developed another minimal medium, which permitted high density growth of lactococci (163). This medium, called ZMB2 was also modified to avoid use of potassium salts in its initial composition, resulting in ZMB2m medium. This time, optical densities above 1.0 were obtained for KCl concentrations between 1.0 and 100 mM in ON cultures of both strains under study (Fig 14, II and III). Hence, ZMB2m medium and KCl concentrations of 0.25, 0.50 and 1 mM were selected to perform the deletion protocol.

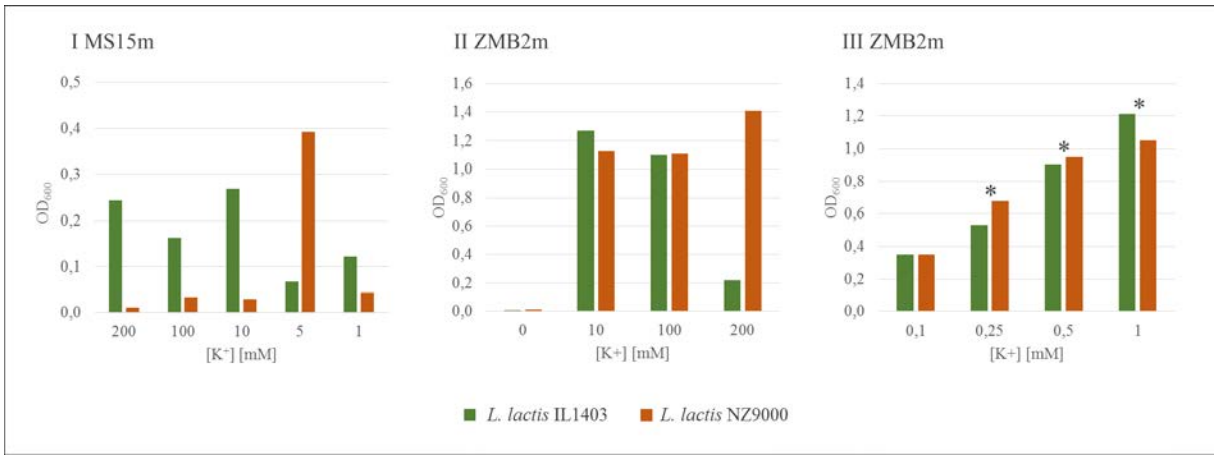

Fig 14 *L. lactis* growth on minimal media. Strains IL1403 and NZ9000 were cultured in modified minimal media without potassium salts. KCl was added prior to inoculation at the concentrations detailed in the graphs. I: Final OD_{600} obtained in MS15m after 5 days. II and III: Final OD_{600} obtained in ZMB2m ON cultures. KCl concentrations used in the deletion protocol are marked with an asterisk.

Even though mutants lacking c-di-AMP synthesizing enzymes were reported to be obtained in minimal media at low K^+ concentrations (49, 60, 62), *cdaA* deletion was not possible for neither of *L. lactis* strains under study in this work. A second approach was also tried, where by use of the nisin inducible pNZ8048 vector, genomic *cdaA* deletion was tried when a *trans* copy was being expressed. In this way, in the absence of nisin, cells would be depleted of CdaA enzymes, and mutants and possible suppressors could be studied. Unfortunately, this strategy was not successful either, for what the focus of this research was now directed towards the modification of c-di-AMP intracellular pools and the obtaining of maximum and minimum levels compatible with growth in rich media for *L. lactis* IL1403.

The starting point to this aim was the development of genetically engineered strains for homologous and heterologous expression of genes encoding c-di-AMP synthesizing and degrading enzymes. Thus, *cdaA* and *gdpP* genes from *L. lactis* IL1403 and *E. faecalis* JH2-2 ($cdaA^{ll}$, $gdpP^{ll}$, $cdaA^{ef}$ and $gdpP^{ef}$, respectively) were cloned in vector pBV153 (Fig 15, I). This vector was developed in our laboratory and it has the Pcit promoter upstream of the multiple cloning site, leaving the gene of interest under pH regulation (164). That is to say, as the pH of the growth medium decreases, expression of the desired protein increases. The resulting plasmids were named pQI101, pQI102, pQI103 and pQI104, respectively and were electroporated in *L. lactis* IL1403 originating strains LL03, LL04, LL05 and LL06, respectively (see Table XV).

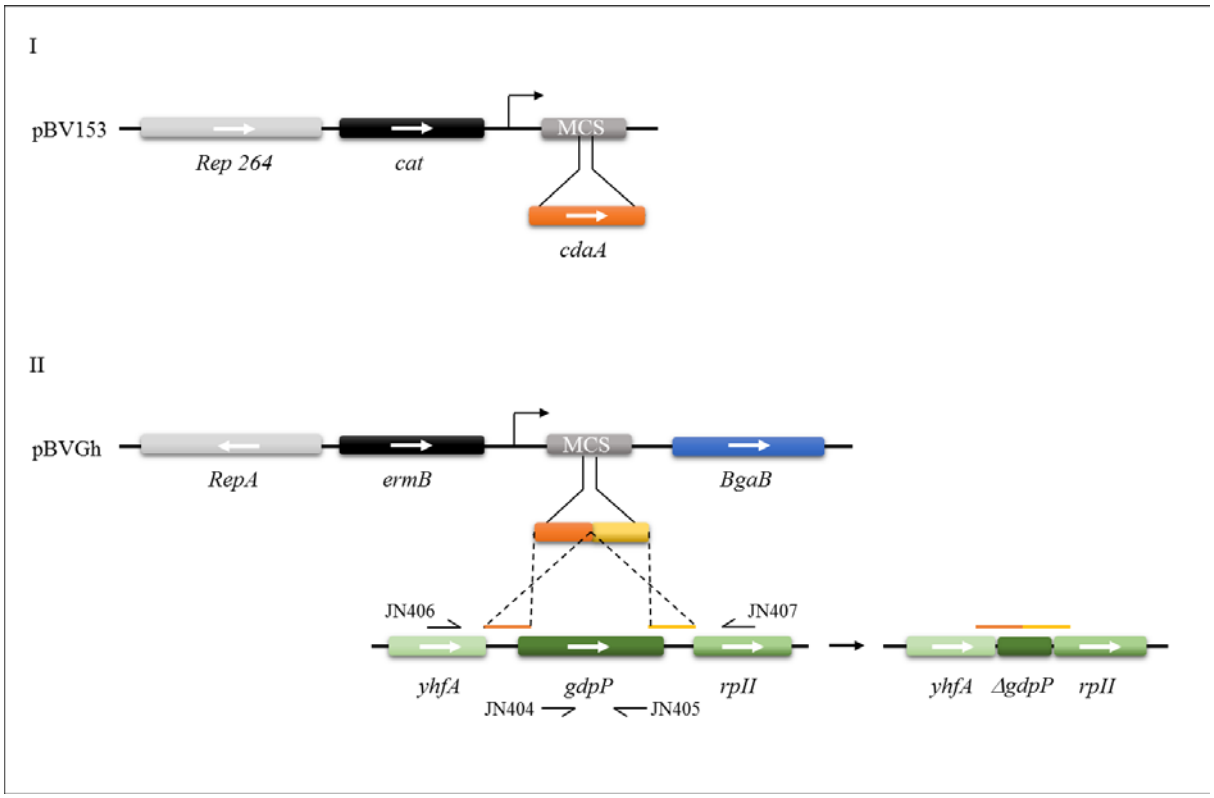

Fig 15 Strain development for modification of c-di-AMP intracellular levels. I: Vector pBV153 was used for homologous and heterologous expression of *cdaA*^{ll}, *gdpP*^{ll}, *cdaA*^{ef} and *gdpP*^{ef}. Only *cdaA* is represented, but the same system was used in a similar way to clone *gdpP* genes as well. II: pBVGh vector employed for gene deletion. This system allows a clean deletion of the gene of interest via a double recombination event. Primers JN404 and JN405 as well as JN406 and JN407 were used for deletion check.

Another strategy considered for modifying intracellular c-di-AMP concentrations was mutation of its degrading enzyme, GdpP. Since this is the only c-di-AMP phosphodiesterase reported in *L. lactis*, deletion of its gene via homologous recombination by use of vector pBVGh was performed (Fig 15, II). The selected thermosensitive vector was previously developed in our laboratory as well, and it allows deletion of the gene of interest via a series of incubations at the permissive and non-permissive temperature, without incorporation of antibiotic resistances in the strain under study (165). Briefly, an up and a downstream region of the gene are adjacently cloned in the plasmid, so homology enables the integration of the plasmid in the chromosome when incubating at the non-permissive temperature. When temperature is lowered, the plasmid jumps out and it carries the gene of interest with it. An easy white/blue colony screen allows identification of putative mutants, which are then checked by antibiotic sensitivity and by two different PCR. The first one is performed with a pair of primers hybridizing outside the fragments cloned (JN406 and JN407 in Fig 15, II), and the second one is a negative control for which primers amplifying a central region of the gene of interest are used (JN404 and JN405 in Fig 15, II).

Once deletion of *gdpP* was confirmed, the selected strains were restricked three times to check colony morphology and growth stability. Interestingly, in this process, two slightly different colony sizes were obtained, for which both samples were kept for later c-di-AMP levels determination. These strains were named *L. lactis* LL07 (smaller colonies) and LL08 (bigger colonies).

3.2.2. Phenotypic analysis of the strains developed

The first analysis of strains constructed was performed on morphology developed in rich media M17G during exponential growth (Fig 16, up) and also once the stationary phase was reached (Fig 16, down). In the first case, the wild type phenotype can be described as short chains of two or four bacteria, with homogeneous and well-defined round shape. This is also true for strains LL05 and LL06, respectively carrying $gdpP^{ll}$ and $gdpP^{ef}$ genes, where no particular phenotype is observed. The main differences, on the other hand, were obtained for strains LL03 and LL04, harboring a *trans* copy of $cdaA^{ll}$ and $cdaA^{ef}$, respectively. In both these cases, a slight increase in cell size can be visualized, as well as no clear division of each cell in dimeric chains. As previously mentioned and as it is widely known, c-di-AMP metabolism is closely related to cell wall biosynthesis, which is why this phenotype would indicate that *cdaA* genes are being expressed.

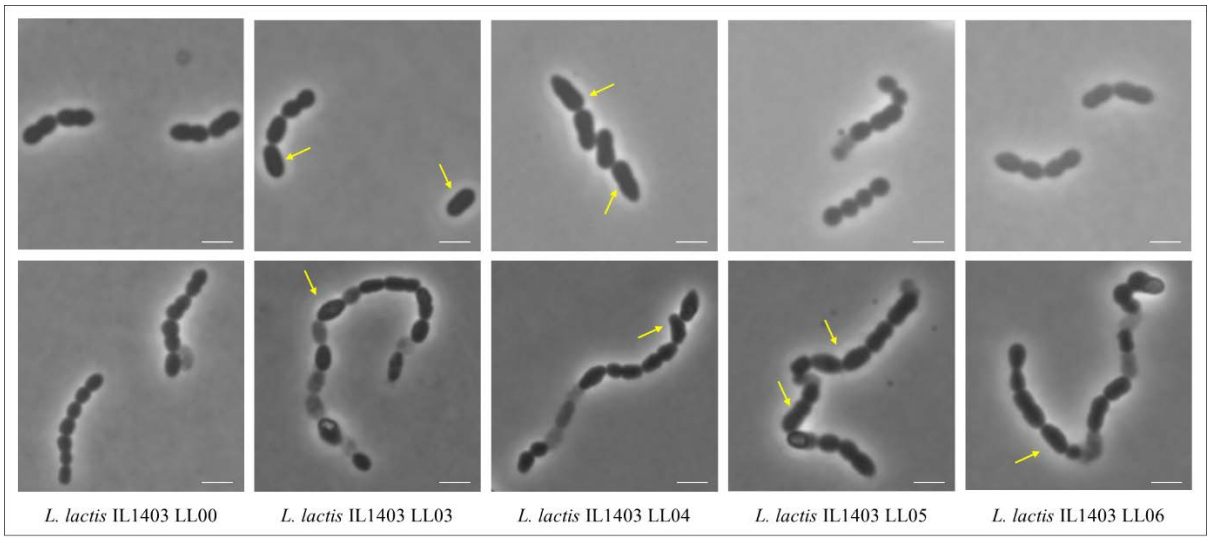

Fig 16 Morphology of *L. lactis* LL00, LL03-LL06 strains. Phase contrast microscopy of strains LL00, LL03-LL06. Bacteria were grown in M17G medium at 30 ºC and samples were taken at mid-exponential phase (up) and at stationary phase (down). Arrows indicate abnormal morphology. Scale bar, 2 μm.

Clearer phenotypes were evidenced for the stationary phase, although they were similar for all strains under study. Control strain LL00 harboring the empty vector formed long cocci shaped strains, whereas strains LL03-LL6 evidenced heterogeneous bacterial sizes and shapes, as well as a higher number of non-viable cells. These results indicate that morphology is more affected in the late exponential phase, probably due to accumulation of c-di-AMP (for strains LL03 and LL04) or long time exposure to low levels of this second messenger (for strains LL05 and LL06).

After analyzing morphology of strains developed to alter c-di-AMP intracellular levels, a second study was done on growth curves obtained in rich media M17G upon addition of different stress factors. Although induction of Pcit promoter increases as the pH of the media decreases, these experiments were performed at initial pH of 7.0 to avoid raising c-di-AMP concentrations into toxic levels. Indeed, the capacity of LAB to acidify media as result of their lactic acid production was ideal to increase induction gradually along growth.

Control curves in M17G grown at 30ºC can be seen in Fig 17, where strain LL03 carrying *cdaA*^{ll} gene, already shows a growth disadvantage with respect to strain LL00 harboring vector pBV153. This detriment is evidenced by a particularly long lag phase, which is enhanced when temperature is raised to 37ºC. Interestingly, Smith et al. reported heat resistant *L. lactis* strains obtained after construction of *gdpP* mutants by use of a protocol which involved an incubation step at 37.5ºC (74). Taking into account that a defective *gdpP* strain and a strain with an extra copy of *cdaA* would have the same effect of increasing c-di-AMP concentration, similar phenotypes were expected. Therefore, the growth defect manifested by strain LL03, could suggest that an even a higher c-di-AMP concentration was reached with respect to the *gdpP* mutants, resulting toxic for the cell, or a mutation in another gene involved in heat resistance.

Strains *L. lactis* LL05 and LL06 (*gdpP*^{ll} and *gdpP*^{ef}, respectively) also evidenced a growth disadvantage, although to a lesser extent than strain LL03.This time, results are in accordance with the heat resistance phenotype of *gdpP* mutants, since an extra copy of the gene would decrease c-di-AMP intracellular pools, causing the opposite effect. Taken together, evidence here presented agree with previous findings about low as well as high concentrations of c-di-AMP being toxic for bacteria (54). Lastly, strain LL04 harboring a *trans* copy of *cdaA*^{ef} gene seems to have no effect on growth, suggesting that it could be inactive in the system here studied.

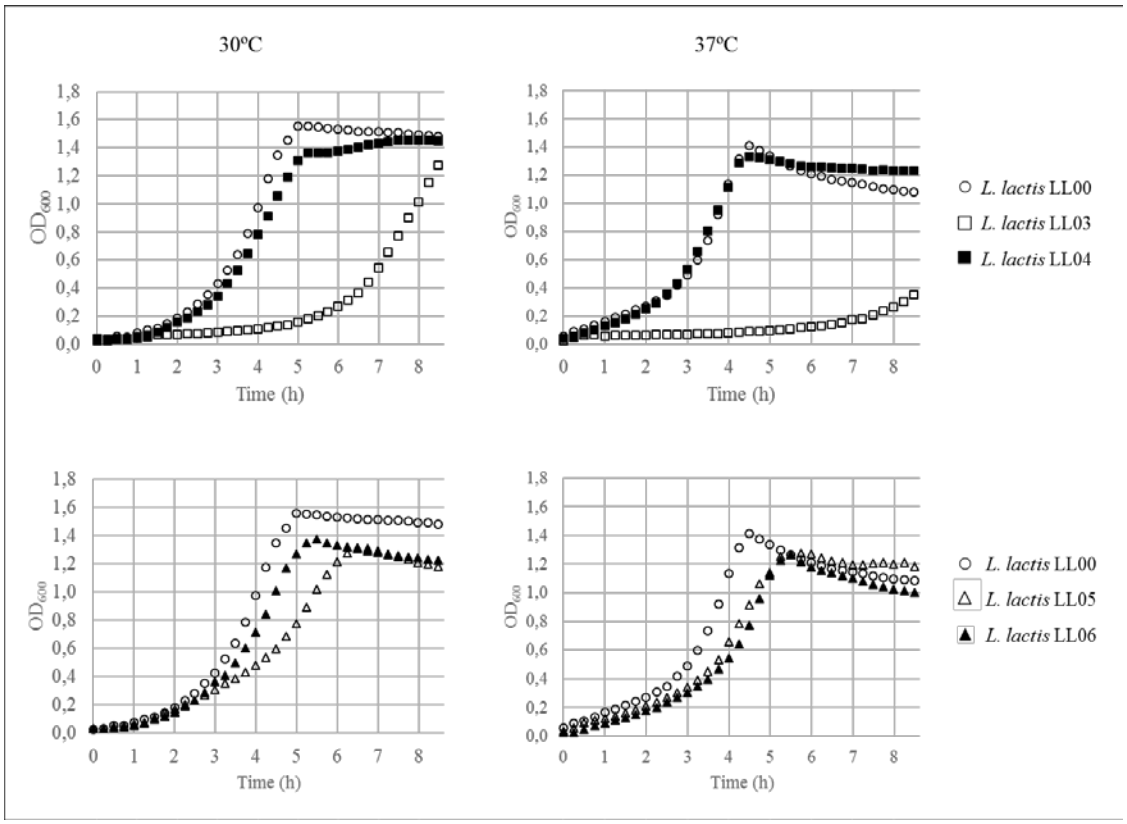

Fig 17 Temperature sensitivity of strains LL00, LL03 – LL06. Growth curves were performed in rich media M17G at 30 ºC (left) and 37 ºC (right). Strain *L. lactis* LL00 harbors the empty vector pBV153, whereas the series LL03, LL04, LL05 and LL06 carry a *trans* copy of genes *cdaA*^{ll}, *cdaA*^{ef}, *gdpP*^{ll}, and *gdpP*^{ef}, respectively.

Moreover, saline hypersensitivity was also reported by Smith et al. for *gdpP* mutant strains of *L. lactis*, and was therefore evaluated for the strains developed in this work (74). Concentrations of 0.25 and 0.50 M NaCl were selected to analyze their impact on growth. As Fig 18 shows, strain LL03 develops an unusually long lag phase of 8 hours and reaches stationary phase after 12 hours. Moreover, when media is supplemented with NaCl 0.50 M, a general detriment in growth is observed, since maximum OD_{600} is lowered, and strain LL03 is not able to grow anymore. On the other hand, strain LL05 (harboring a *trans* copy of *gdpP*^{ll}) also shows impaired growth, although the effect is not as marked as the one just described. Conversely, neither the presence of *cdaA*^{ef} in strain LL04, nor of *gdpP*^{ef} in strain LL06seems to have an evident impact on growth under these conditions, since in both cases growth is similar to strain LL00, carrying the empty vector.

Impact of β-lactam antibiotics on strains under study was also tested. These are a class of antibiotics active against a group of proteins called “penicillin-binding proteins” or PBP. A subgroup of essential PBP are the transpeptidases, which are serine proteases in charge of the last step in cell wall biosynthesis: the cross-linking of neighboring peptidoglycan strands (166).

The β-lactam ring of these antibiotics mimics the cell wall strand, but once in contact with the enzyme, it forms a stable intermediate that inactivates the transpeptidase. Consequently, an imbalance occurs between the incorporation of new building blocks to the cell wall and the recycling activity of autolysins, leading to cell lysis (167).

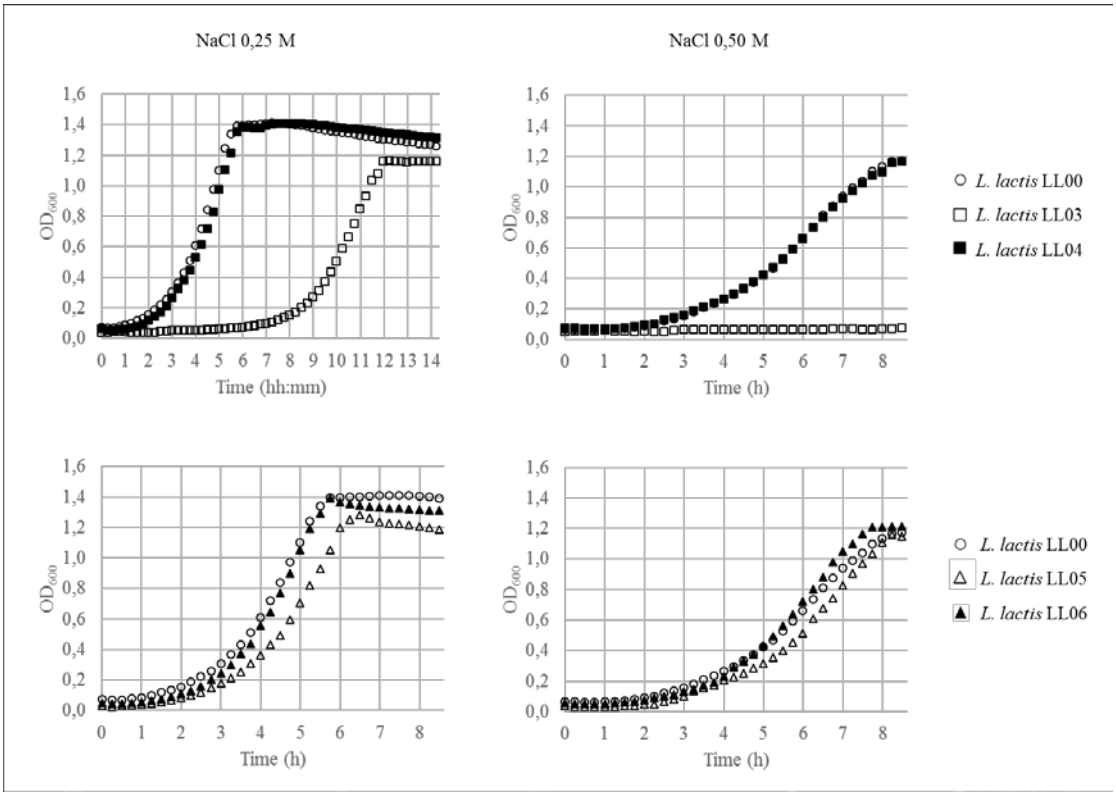

Fig 18 Saline sensitivity of strains LL00, LL03 – LL06. Growth curves were performed at 30 ºC in rich media M17G supplemented with 0.25 M (left) and 0.50 M (right) NaCl. Strain *L. lactis* LL00 harbors the empty vector pBV153, whereas the series LL03, LL04, LL05 and LL06 carry a *trans* copy of genes $cdaA^{ll}$, $gdpP^{ll}$, $cdaA^{ef}$ and $gdpP^{ef}$, respectively.

c-di-AMP homeostasis has been reported to be connected to cell wall metabolism, and in fact, CdaA is modulated by GlmM, an enzyme in charge of synthesizing the cell wall precursor glucosamine-1-phosphate (82). Moreover, *gdpP* mutants in *L. lactis* have also been found to be more resistant to sublethal concentrations of penicillin G (74). Taking this into account, response to sublethal concentrations of penicillin and ampicillin (two β-lactam antibiotics) was tested for strains LL03-06.

Once more, no significant effect was observed for strain LL04 and a growth disadvantage was evidenced for strain LL03 when media were supplemented with 0.25 μg/ml ampicillin or 0.10 μg/ml penicillin, showing a more severe effect for the latter case. On the other hand, even though both strains LL05 and LL06 manifested impaired growth in presence

of 0.25 μg/ml ampicillin, strain LL06 grew more similar to the LL00 control strain when 0.10 μg/ml penicillin was added to the medium instead Fig 19.

So far, only the response to β-lactam antibiotics has been reported for *L. lactis* strains with mutations altering c-di-AMP intracellular levels. Since response to different stress factors could also allow the identification of other pathways related to c-di-AMP metabolism, growth curves in presence of compounds with a different mechanism of action was evaluated.

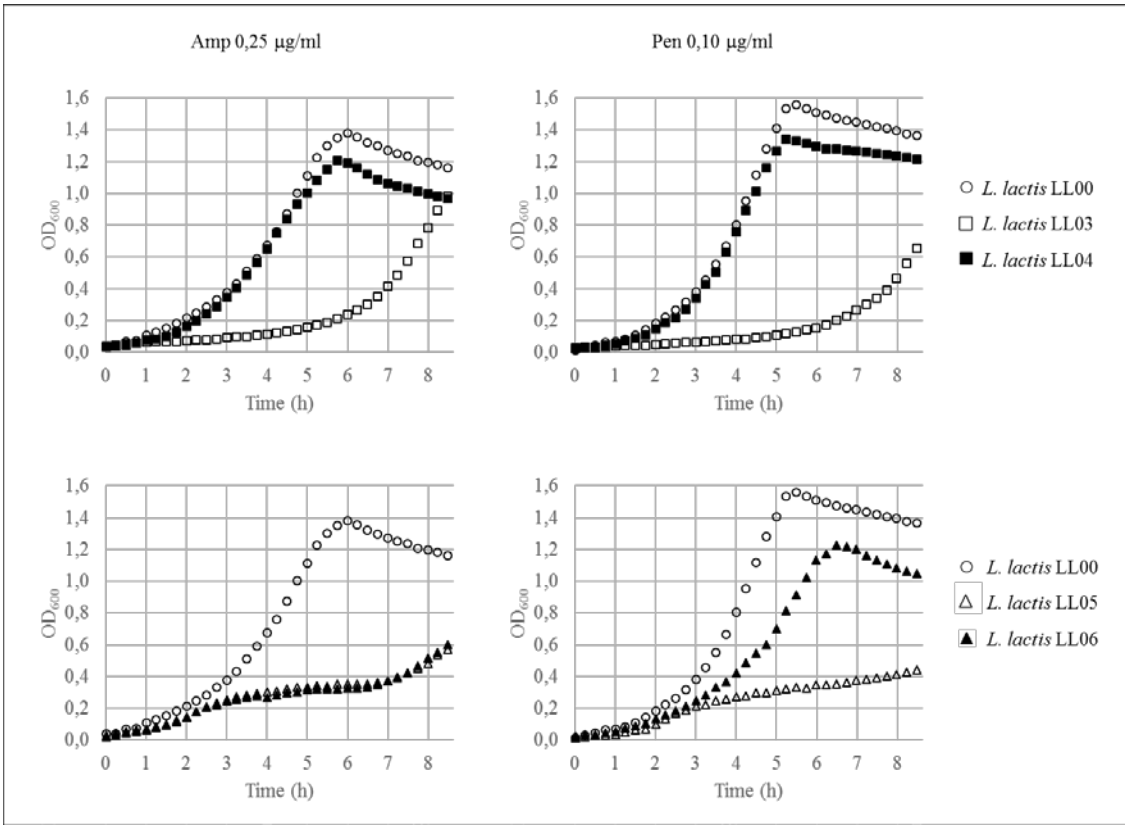

Fig 19 Response of strains LL00, LL03 – LL06 to β-lactamic antibiotics. Growth curves were performed at 30 ºC in rich media M17G supplemented with 0.25 μg/ml ampicillin (left) and 0.10 μg/ml penicillin (right). *L. lactis* LL00 harbors the empty vector pBV153, whereas the series LL03, LL04, LL05 and LL06 carry a *trans* copy of genes $cdaA^{ll}$, $gdpP^{ll}$, $cdaA^{ef}$ and $gdpP^{ef}$, respectively.

Vancomycin is a glycopeptide active against the second phase of cell wall synthesis, i.e. it binds to D-Ala-D-Ala ends of monomers being processed for its later addition to the cell wall. Nevertheless, once vancomycin is bound, the complex formed is no longer a substrate for subsequent enzymes and consequently lipid intermediates accumulate in the membrane (168). Considering that it affects a different stage of cell wall biosynthesis, its impact on cells with modified intracellular pool of c-di-AMP could give insights as regards the importance of this second messenger in the different stages.

As Fig 20 shows, only strains LL03 and LL05, harboring an extra copy of *cdaA*^{ll} and *gdpP*^{ll}, respectively, evidenced a negative impact on growth, when media were supplemented with 0.50 μg/ml vancomycin. *E. faecalis* enzymes seem not to affect the response, having a behavior similar to the control with the empty vector.

On the other hand, lysozyme is an enzyme capable of hydrolyzing the glycosidic bonds on the cell wall, releasing a disaccharide formed by acetylglucosamine and muramic acid (169). Its impact as an antimicrobial agent has long been studied, and it has also been reported that in *L. monocytogenes* the lack of CdaR increased resistance to this compound (170). Taking into account that in the same work it was proved that CdaR negatively regulates CdaA in this bacterium, this would mean that high amounts of c-di-AMP could favor lysozyme resistance. Therefore, this hypothesis was tested for the strains here developed.

In Fig 20, it can be observed that an extra copy of *gdpP* gene (LL05 and LL06) increased susceptibility to lysozyme. At a concentration of 0.10 μg/ml, LL05 and LL06 (*gdpP*^{ll} and *gdpP*^{ef}, respectively) strains are no longer able to grow in the time interval here presented, whereas the control strain reaches a final OD_{600} of 1.0. Taking into account that both strains are expected to produce higher amounts of GdpP proteins, and consequently lower their c-di-AMP levels inside the cell, these results agree with the ones previously described for *L. monocytogenes*.

Interestingly, different phenotypes were evidenced for strains harboring an extra copy of *cdaA*^{ll} and *cdaA*^{ef}. Even though *trans*-expression of the former impedes growth of strain LL03, the phenotype observed due to expression of latter in strain LL04, agrees with the published results mentioned, since it confers a growth advantage with respect to the control LL00 strain, harboring the empty vector.

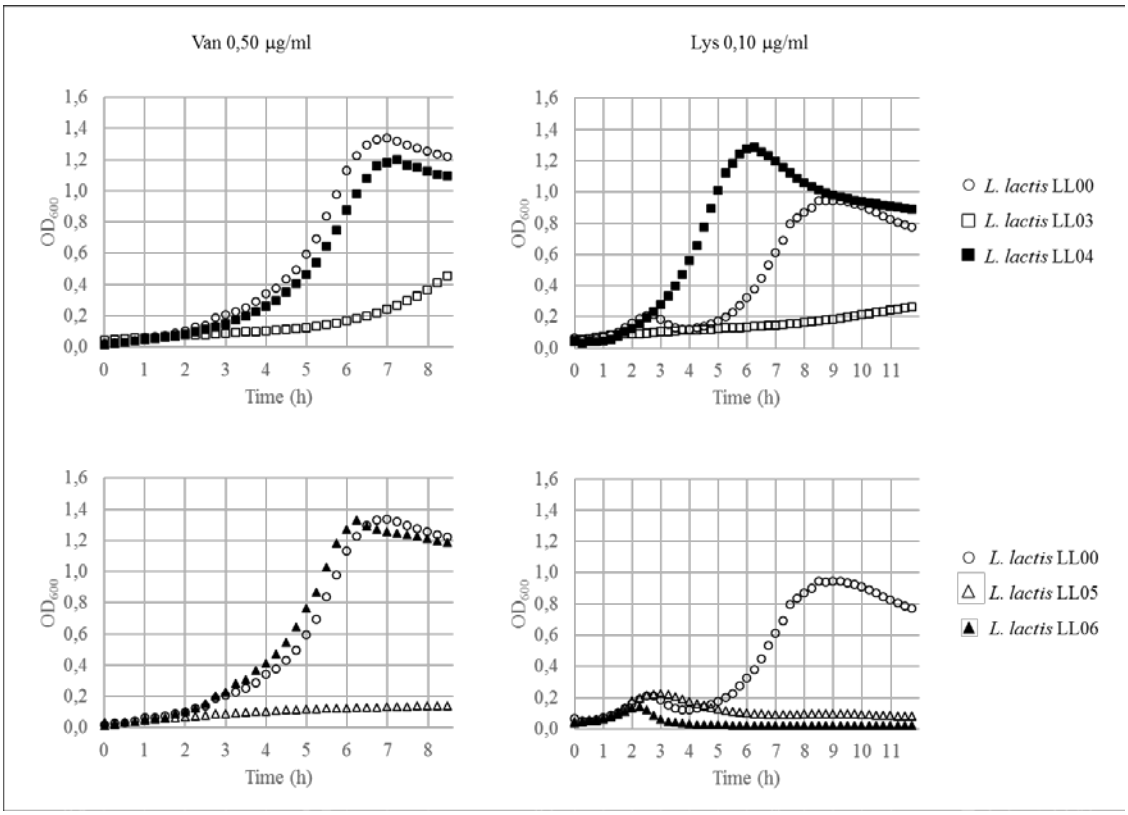

Fig 20 Impact of vancomycin and lysozyme on strains LL00, LL03 – LL06. Growth curves were performed at 30 ºC in rich media M17G supplemented with 0.50 µg/ml vancomycin (left) and 0.10 µg/ml lysozyme (right). *L. lactis* LL00 harbors the empty vector pBV153, whereas the series LL03, LL04, LL05 and LL06 carry a *trans* copy of genes $cdaA^{ll}$, $gdpP^{ll}$, $cdaA^{ef}$ and $gdpP^{ef}$, respectively.

Finally, the two isolated *gdpP* mutants (strains LL07 and LL08) were also analyzed by phase contrast microscopy in order to evaluate differences in morphology. In Fig 21, images of bacteria during mid-exponential growth are presented (down), where a slight decrease in size can be seen for strain LL07, which showed smaller colonies. In addition, for both samples under study, well defined round shaped cells were evidenced, with no significant differences with respect to the control strain LL00 harboring the empty vector pBV153.

As previously mentioned, *gdpP* mutants analyzed by Smith et al. showed improved growth at sublethal concentrations of the β-lactamic antibiotic penicillin. Therefore, both mutant strains LL07 and LL08 were grown in presence of ampicillin to further study possible phenotypic differences between them. In Fig 21, growth curves obtained in M17G supplemented with 5.0 µg/ml ampicillin are presented, where a detriment is evidenced for both strains. Interestingly, the opposite phenotype was observed in these conditions, and even a slight difference is observed in *ΔgdpP* strains LL07 and LL08, being the former more sensitive to the antibiotic than the latter.

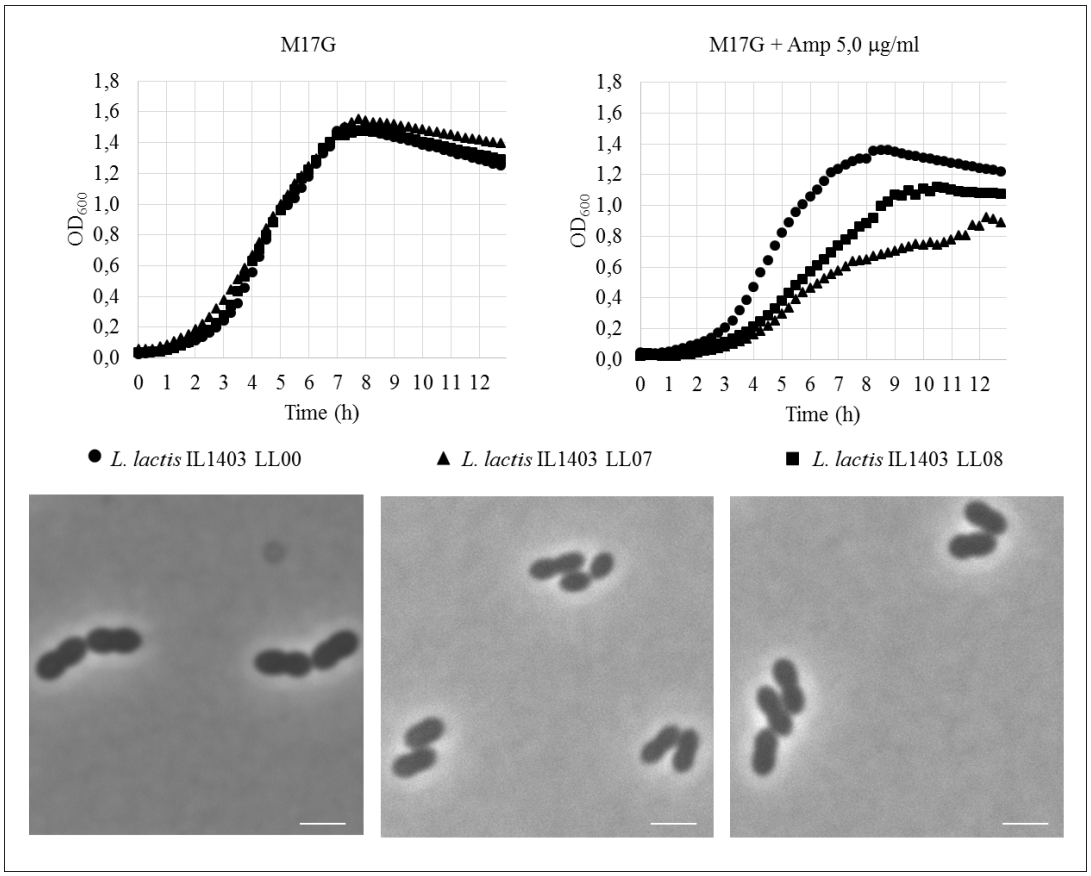

Fig 21 Phenotypic analysis of *ΔgdpP* mutants Up: Growth curves performed in rich media M17G without (left) and with supplementation of 5.0 µg/ml ampicillin (right). Down: Phase contrast microscopy of strains LL00, LL07 and LL08. Bacteria were grown in M17G medium at 30 ºC and samples were taken at mid-exponential phase. Scale bar, 2 µm.

3.2.3. Intracellular levels of c-di-AMP are modified in strains *L. lactis* LL03-LL08

After analyzing phenotypes and stability of strains developed in the previous section, their c-di-AMP intracellular levels were determined. As mentioned, pBV153 vector is induced at low pH, which is why strains LL00, LL03-06 harboring this plasmid and its derivatives were grown at initial pH (pH_0) of 7.0 and 5.5 for later c-di-AMP quantification. Briefly, cells were grown in M17G supplemented with 10 µg/ml Cm for strains LL00, LL03-06 and without antibiotic for strains LL07 and LL08. OD_{600} measurements were taken every hour to check growth (not shown). When OD_{600} reached 0.5, cells were harvested and pellets stored for c-di-AMP extraction and determination. Results were normalized by the amount of protein present in the samples (see Materials and Methods).

Values obtained for strain LL00 as well as IL1403 indicate that basal levels of c-di-AMP are in the range of 19.9 – 41.3 and 28.0 – 34.1 ng of c-di-AMP per mg of protein at pH_0

7.0 and 5.5, respectively. These levels could be significantly increased when strain LL03 with plasmid pIQ101 harboring *cdaA*^{ll} gene was grown at pH_0 = 7.0. Average values obtained in these conditions, for three technical replicates were 334.0 and 350.0 ng of c-di-AMP per mg of protein in biological duplicates (Fig 22). This means that at least 8 times higher values are possible to obtain in *L. lactis* IL1403 without reaching toxic levels. Furthermore, when plasmid was pIQ101 was induced at pH_0 5.5, c-di-AMP concentrations were almost doubled reaching average values of 698.4 and 652.5 ng of c-di-AMP per mg of protein in biological duplicates, more than 15 times higher than basal levels just mentioned.

Accordingly to results described in the previous section, where strain LL04 with plasmid pIQ102 harboring a copy of *cdaA*^{ef} showed no particular phenotypes when grown in presence of different stress factors, no impact on c-di-AMP levels was manifested in this experiment either. At both pH_0 similar values to the ones observed for the control strain LL00 were obtained, suggesting once more that CdaA^{ef} enzyme is not active under the working conditions of this study.

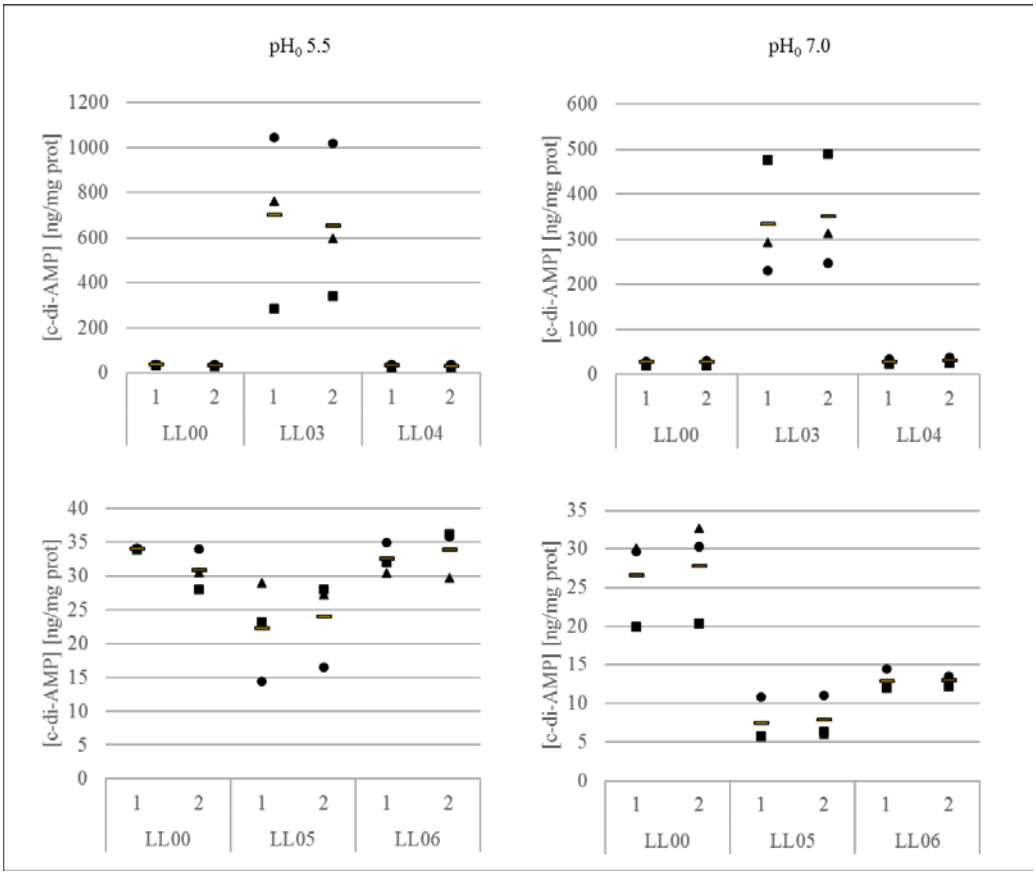

Fig 22. c-di-AMP intracellular levels of strains LL00, LL03-06. Cells were grown in rich M17G medium and harvested at OD = 0.5 for c-di-AMP extraction and quantification. Strains were grown at pH_0 = 5.5 (left) and 7.0 (right). Circles, squares and triangles correspond to technical triplicates. 1 and 2 indicate biological duplicates.

On the other hand, the presence of a *trans* copy of *gpdP*^{ll} gene in strain LL05 allowed reduction of intracellular c-di-AMP values to 14.4 – 29.0 and 5.7 – 11.0 ng of c-di-AMP per mg of protein at pH_0 of 5.5 and 7.0, respectively. These values indicate that the enzyme could be more active at pH_0 7.0, and coincide with the ones obtained for strain LL06, which were also lower at pH_0 7.0, although results above described suggest a higher induction of the expression system at pH_0 5.5.

Interestingly, different results were obtained for the two *gdpP* mutant strains LL07 and LL08. In the first case, values in the range of wild type strain IL1403 were registered (Fig 23), whereas in the second case, intracellular c-di-AMP concentrations were almost doubled (63.6 – 85.1 ng of c-di-AMP per mg of protein). It is important to mention that the fact that c-di-AMP levels are held close to wild type ones even in the absence of *gdpP*, suggests that there could be another c-di-AMP degrading enzyme encoded in *L. lactis* genome.

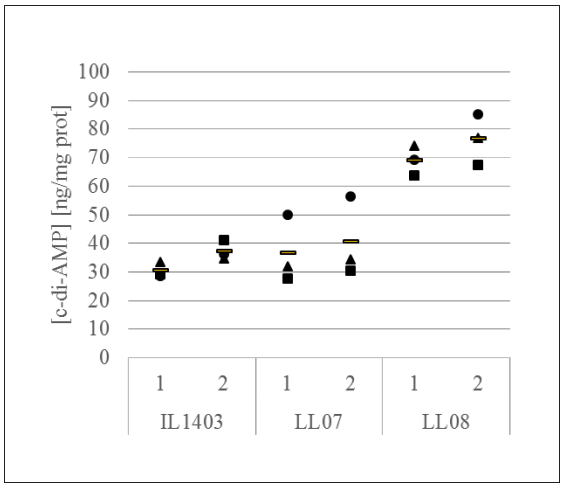

Fig. 24 Intracellular concentrations of c-di-AMP in *L. lactis* Δ*gdpP* mutants. Strains were grown at pH_0 = 7.0 and haversted when OD_{600} reached 0.5. Different c-di-AMP values were obtained for both *gdpP* mutant strains. Circles, squares and triangles correspond to technical triplicates. 1 and 2 indicate biological duplicates

Finally, the impact of ampicillin on c-di-AMP levels was analyzed for strains LL05 and LL06. Both were grown in rich media M17G in presence of 0.25 μg/ml and since LL06 in the previous section evidenced more resistance to β-lactamic antibiotics, a concentration of 0.5 μg/ml was selected in this case as well. Fig. 25 shows that ampicillin supplementation of media causes a decrease of c-di-AMP levels, being the lowest concentration in the range 3.7 – 12.4 ng of c-di-AMP per mg of protein at pH_0 7.0 for strain LL06, harboring a *trans* copy of *cdaA*^{ef} gene.

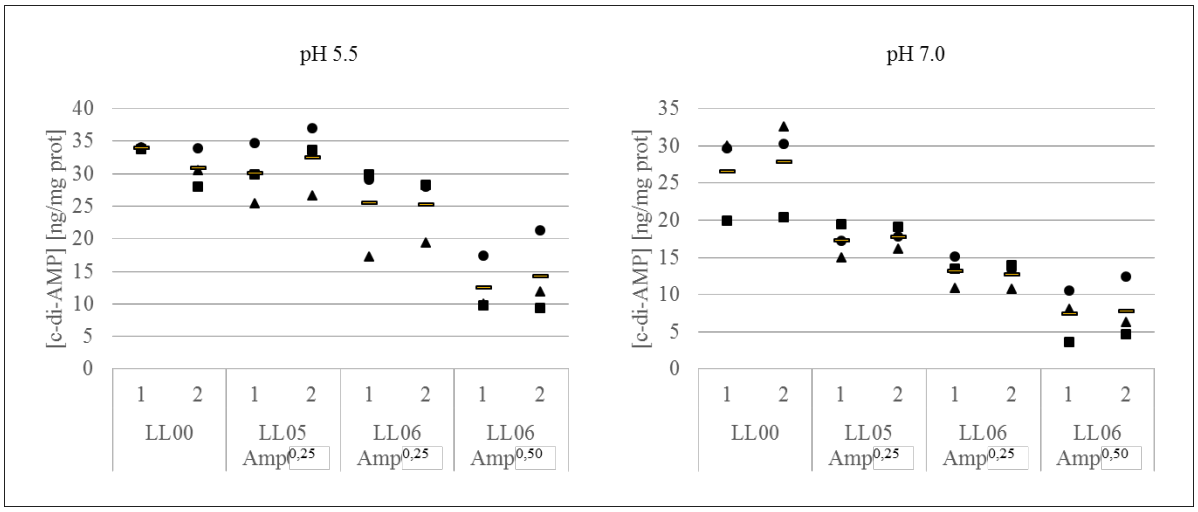

Fig. 25 Ampicillin impact on strains *L. lactis* LL05 and LL06. Strains were grown in in rich medium M17G at pH_0 = 5.5 (left) and pH_0 = 7.0 (right). c-di-AMP levels were taken at OD_{600} = 0.5. The lowest c-di-AMP levels are seen for strain LL06 at pH_0 = 7.0. Circles, squares and triangles correspond to technical triplicates. 1 and 2 indicate biological duplicates.

Altogether, these results allowed us to establish working conditions where c-di-AMP are increased more than 15 times. Therefore, for the immunological experiments in the following chapter, strain LL03 as well as the system of expression with pIQ101 vector induced at pH_0 = 5.50 were selected.

3.2.4. Phosphodiesterases c-di-AMP in *L. lactis*

In the previous section, the maintenance of wild type c-di-AMP intracellular levels in *ΔgdpP* mutant strains suggests the possibility of other enzymes encoded in *L. lactis* genomes capable of degrading this second messenger. As mentioned in the introduction, the second family of c-di-AMP phosphodiesterases, which comprises *B. subtilis* PgpH, contains an HD-domain and a His-Asp motif. Even though homologues are found in many members of firmicutes, a protein blast in *L. lactis* using this protein as query, threw no positive results.

Another group of smaller phosphodiesterases presents only a soluble DHH/DHHA1 domain and was found for example in *S. pneumoniae* and *B. subtilis*, although in the latter this enzyme does not degrade c-di-AMP. This time the protein threw a protein encoded by *yheB* of *L. lactis* IL1403 with 48% identity to *B. subtilis* NrnA and 64% to *S. pneumoniae* Pde2, albeit no further information about its function is available.

With the aim of starting a characterization of YheB protein and evaluating whether it can degrade c-di-AMP, *yheB* gene from *L. lactis* IL1403 and NZ9000 was cloned in the

expression vector pWH844 (see Table XVI Plasmids). This vector allows *E. coli* overexpression of heterologous genes with an N-terminal His_{6X}-tag for purification purposes. Moreover, GdpP proteins from both strains were cloned as well to be used as positive control of c-di-AMP hydrolyzing enzymes, although these experiments could not be performed due to time restraints. Resulting plasmids pIQ401, pIQ402, pIQ403 and pIQ404 containing genes $yheB^{NZ}$, $gdpP^{NZ}$, $yheB^{IL}$ and $gdpP^{IL}$ were electroporated in *E. coli* BL21, originating strains *E. coli* YB01, GP01, YB02 and GP02, respectively.

After checking all gene sequences, an expression test was performed in rich LB medium at 37º. Briefly, strains were grown until OD_{600} reached 0.5, when 0.5 mM IPTG was added and cultures were incubated for three more hours. Fig 26 shows an SDS-PAGE were bands of circa 35 kDa and 74 kDa evidence the presence of YheB and GdpP for strains YB01 – 02 and GP 01-02, respectively.

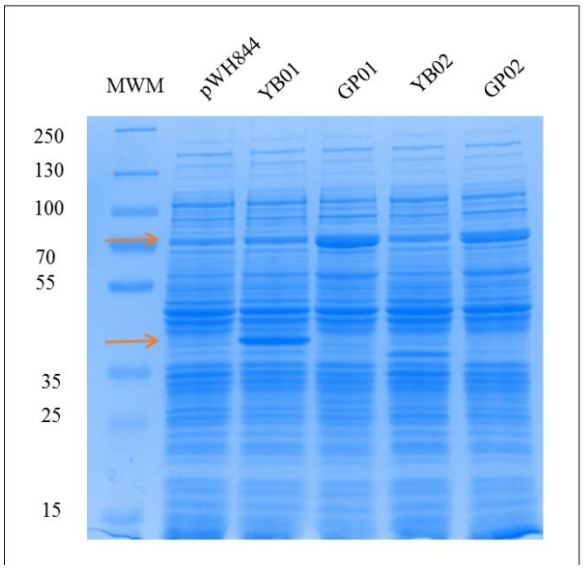

Fig 26 Expression test of strains *E. coli* YB01 -02 and GP01 -02. SDS-PAGE gel after Coomasie staining. Arrows indicate the presence of expression bands corresponding to proteins $YheB^{NZ}$ and $YheB^{IL}$ in lanes 2 and 4, respectively as well as $GdpP^{NZ}$ and $GdpP^{IL}$ in lanes 3 and 5, respectively. MWM: molecular weight marker.

Once production of proteins was confirmed, both YheB proteins were purified using a Ni^{2+}-NTA resin (see Materials and Methods). Fig 27 shows SDS-PAGE gels were the purification process is evidenced and the proteins of interest are present in fractions E3 and E4 for $YheB^{IL}$ (left) and E2, E3 and E4 for $YheB^{NZ}$ (right). Afterwards, proteins were further purified via size exclusion chromatography (SEC) by use of the Äkta system to eliminate imidazole of the media. Samples were then concentrated and frozen in liquid nitrogen to be finally stored at -80º C until used.

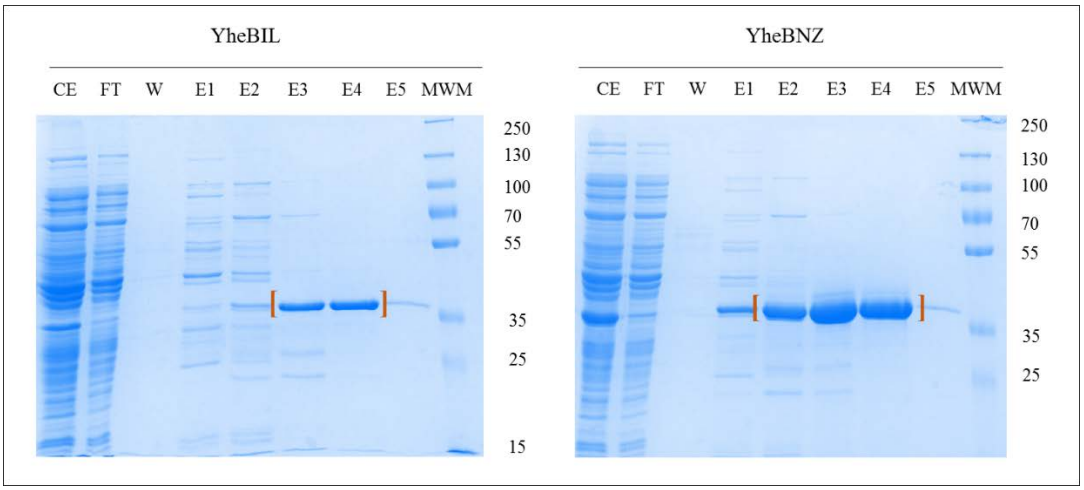

Fig 27 Purification of YheB^{IL} and YheB^{NZ} proteins. Elutions further purified via size exclusion chromatography are highlighted in orange. CE: crude extract, FT: Flow through, E: elution, MWM: molecular weight marker. E1 – E5: 30 – 50 – 100 – 200 – 500 mM imidazole, respectively.

A first approach into function determination of YheB proteins was performed via the BNPP assay. bis(4-nitrophenyl)phosphate can be cleaved by phosphodiesterases yielding paranitrophenol, which production can be followed colorimetrically at 410 nm ((171), Materials and Methods). Reactions were performed at different pH and adding Mn^{2+} to the media to analyze optimal conditions for further studies. As seen in Fig 28, YheB^{NZ} enzyme seems to be more active, and both proteins evidenced increased activity when pH was raised. These results suggest that YheB protein could be a c-di-AMP phosphodiesterase.

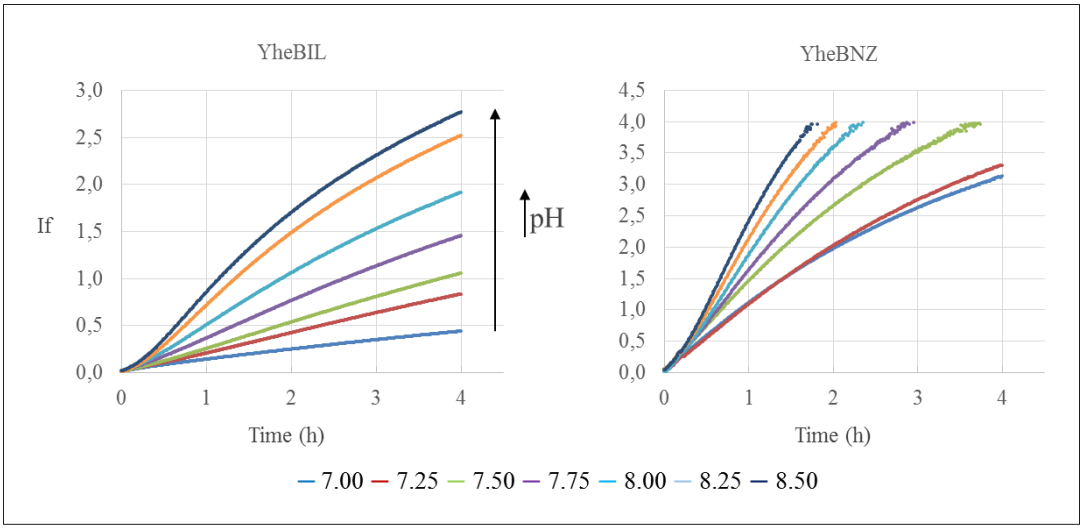

Fig 28 BNPP assay for YheB proteins. YheB^{IL} (left) and YheB^{NZ} (right) were analyzed via BNPP assay. Reads were performed for four hours in a microplate reader. The arrow shows increasing pH in the reaction media. Both graphs show higher activity when pH is raised.

3.2.5. Conclusions

The aim of this chapter was to evaluate the physiological consequences when c-di-AMP intracellular pools are altered in *L. lactis* IL1403. Depletion of this secondary messenger via *cdaA* deletion was not possible in rich, nor in minimal media. The strategy was thus changed and responses obtained along the different experiments here presented, evidenced overexpression of heterologous and homologous *cdaA* and *gdpP* genes by use of pBV153 vector.

In general, strains here developed were more sensitive on rich media and upon the presence of stress factors such as NaCl, antibiotics and high temperature. The only exception was *cdaA*^{ef} overexpressed in presence of 0.1 μg/ml lysozyme, which provided a growth advantage. Since the resulting CdaA^{EF} enzyme showed no great impact on c-di-AMP levels, it could be said that in this conditions, its activity is diminished. If this is the case, detrimental phenotypes obtained in the other growth curves could be due to a system were c-di-AMP levels are too low or too high, which has already been reported to result in impaired growth (172).

The highest average values of c-di-AMP obtained in this work reached 698.4 and 652.5 ng of c-di-AMP per mg of protein in biological duplicates for strain LL03. Although these differences could be due to methodological variations, an increment of more than 15 times with respect to basal levels was obtained. *L. lactis* LL03 was therefore selected for further studies on the development of a live vaccine prototype in next chapter.

On the other hand, reduction of c-di-AMP intracellular levels by expression of *gdpP* genes was more significant at pH_0 7.0. Despite the fact that the induction of the expression system used is higher when pH is lowered, these results lead to the hypothesis that GdpP enzyme activity in *L. lactis* would prevail when extracellular pH is closer to physiological values. Moreover, the biggest impact in this way was accomplished by strain LL06 grown in rich media M17G supplemented with 0.50 μg/ml ampicillin. Average values of technical triplicates were 7.4 and 7.8 ng of c-di-AMP per mg of protein, linking the impact of a β-lactamic antibiotic to c-di-AMP levels in *L. lactis* IL1403.

Finally, the fact that *ΔgdpP* mutants are able to maintain c-di-AMP levels close to wild type ones, supports the hypothesis of the existence of at least another enzyme capable of hydrolyzing c-di-AMP. The main candidate found in this work is the uncharacterized YheB protein, which was proved here to degrade BNPP. Nevertheless, this compound is an unspecific

substrate for phosphodiesterases, and even though phosphodiesterase activity is evidenced here, and proved to increase at higher pH, further specific studies are needed to confirm it can degrade c-di-AMP (68).

.

3.3. Development of an oral subunit vaccine prototype against Chagas disease

3.3.1. Antigen design and production in *L. lactis*

As mentioned in the introduction, *T. cruzi* trans-sialidase (TS) enzyme plays a vital role upon infection of the mammal host, since it catalyzes the transfer of sialic acid from the host glycoconjugates to the terminal β-galactopyranosyl residues of mucin-like molecules on the parasite's cell surface. It is also involved in different pathways leading to successful parasite infection and down-regulation of the host immune response (173). Therefore, TS was selected to develop a suitable antigen for the vaccine prototype against Chagas disease.

The high performance of the whole TS protein as a *T. cruzi* antigen in vaccine prototypes has already been reported (174, 175). Nevertheless, considering that protein overexpression in LAB is not as straight forward as normally is in other bacterial models such as *E. coli*, an abbreviated fraction of the antigen was selected instead of the entire TS protein. In addition, the engineered *L. lactis* strain was designed to co-express the synthesizing enzyme of the adjuvant as well, which adds a detrimental factor to bacterial growth and protein expression. Altogether, in order to facilitate protein co-expression in *L. lactis*, the smallest TS protein size possible was selected, having at the same time the highest epitope concentration able to trigger a TS-specific immune response.

Based on these criteria, an epitope prediction within the complete TS antigen sequence was performed by Ivan Marcipar group in Laboratorio de Tecnología Inmunológica, Facultad de Bioquímica y Ciencias Biológicas, UNL (Universidad Nacional del Litoral). T epitopes against H-2Kd MHC-I were selected for prediction, and a total of 7 epitopes were identified, with four of them in the central region of the protein, ranging from amino acid 326 to 497. Interestingly, IYNVGQVSI epitope was included in this region, which has been described as the main MHC-I T-cell epitope that provides protection against *T. cruzi* infections in BALB/c mice (176, 177). On the other hand, B-epitopes have shown to comprise key amino acids responsible for enzymatic catalysis (178), which is why antibodies developed against this region may potentially neutralize TS activity. Therefore, prediction of B epitopes was also carried out, identifying 15 out of the 50 epitopes with the highest score within the same central

region. These predictions lead to the final TS fragment selection, ranging from amino acid 326 to 497 (Fig. 29).

Given that synthesis of heterologous proteins may result in amino acid unbalance, the designed peptide was submitted to GenScript synthesis service (U.S.A.) to optimize codon usage for *L. lactis* and to incorporate *Nco*I and *Hind*III restriction sites required for cloning, as well as an N-terminal His_{6x} tag to allow detection by Western blot. The resulting gene was named *tscf* and cloned into the nisin inducible pNZ8048 vector previously described (pNZTS, Table XVI, Fig. 29), to be finally electroporated in *L. lactis* NZ9000 (strain NZTS, Table XV). After checking the genetic construction by sequencing, several conditions of expression were tested. Protein overproduction was proved in some of them, but it was not possible to detect it again in new trials of expression (not shown).

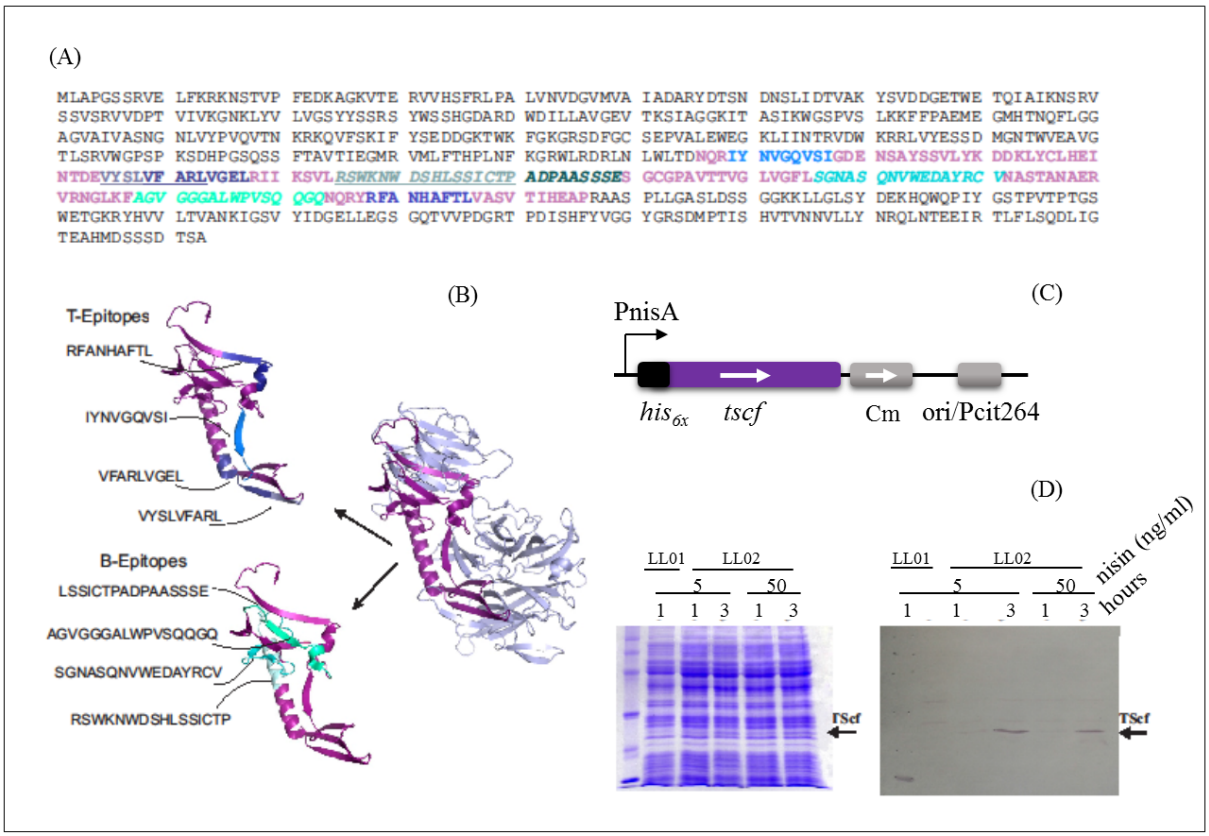

Fig. 29 Development of an optimized TS derived antigen. A: complete sequence of trans-sialidase protein. The selected fragment is highlighted in purple and colored amino acids within this sequence indicate epitopes. Underlined amino acids refer to superimposed epitopes. B: Structure modelling of whole TS (right) and the two sections conforming the synthetic antigen (left). C: cloning representation of his_{6x}-*tscf* in plasmid pNZ8048. D: Expression check by SDS-PAGE (left) and Western blot analysis (right).

As mentioned in the introduction, *L. lactis clpP-htrA* strain favors expression of heterologous genes since it lacks the two major proteases ClpP and HtrA, reducing degradation

of heterologous proteins. Plasmid pNZTS was electroporated in this strain, resulting in strain LL02 (Table XV), and this time stable overproduction of TScf peptide was obtained. Briefly, cells were grown in 3 liters of M17G broth at 30 ºC until OD_{600} reached 0.50 and induced with 5 or 50 ng/ml nisin during 1 or 3 hours. As shown in Fig. 29, Western blot analysis using anti-his antibodies allowed visualization of a clear band when cultures were induced for 3 hours. Protein identity was determined by mass spectrometry (MS/MS), confirming overproduction of TScf.

3.3.2. Immune response after mucosal co-administration of *L. lactis* strains producing TScf antigen and c-di-AMP adjuvant

Once a strain able to produce the designed antigen (LL02) as well as a strain synthesizing high adjuvant levels (Chapter II, strain LL03) were obtained, the next step was to evaluate the effectiveness of their co-administration, as proof of concept for the development of a new prototype of mucosal vaccine. In collaboration with Dra. Ana Rosa Perez's group, from the Instituto de Investigación Clínica y Experimental de Rosario (IDICER CONICET-UNR), three successive oral immunizations were performed in mice (see Materials and Methods). Studied groups were: Non-immunized (NI, mice receiving only PBS); LL02, mice immunized with bacteria producing the TScf antigen (*L. lactis* LL02, Table XV); LL02+LL03 group, mice co-administered with *L. lactis* LL02 plus bacteria over-producing c-di-AMP (*L. lactis* LL03, Table XV). *L. lactis* LL01 strain harboring the empty pNZ8048 plasmid was also orally administered as control. In addition, since earlier studies showed a higher specific anti-TS cellular response after immunization with TS plus ISCOMATRIX adjuvant by subcutaneous immunization (174), for comparative purposes a group of mice was also immunized with purified TScf adjuvanted with ISPA (C+, Gold standard group), an ISCOMATRIX type adjuvant developed by Dr. Marcipar group (152).

As shown in Fig. 30, 15 days after the last immunization, 48 hours footpad testing showed that *L. lactis* LL02+LL03 immunized group elicited a clear TS-specific cell-mediated immune response compared to PBS and LL01, while this difference is less significant compared with the *L. lactis* LL02 group. Interestingly, the increase in footpad thickness were similar between *L. lactis* LL02+LL03 and the Gold standard group. These results support that TScf sequence contains MHC-I T-cell epitopes, as was previously mentioned, but also suggest that

orally-administered *L. lactis* over-expressing *cdaA* gene (LL03) could be used as immune stimulator of the response against this antigen.

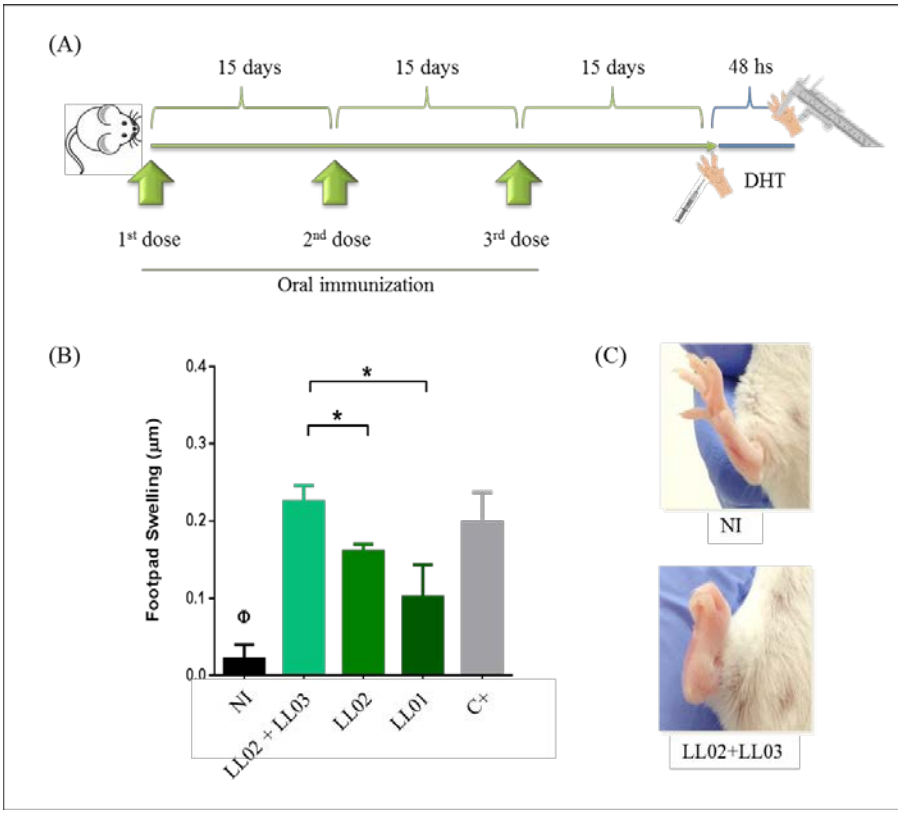

Fig. 30 Graphic representation of BALB/C mice oral immunization protocol. A: Oral immunization scheme. Three doses were administered with 15 days intervals. 48 hours after the last immunization, footpad swelling and DHT were measured. B: Co-administration of LL02+LL03 shows a significant difference with respect to LL02 as well as LL01 groups. C: Morphological difference in footpad swelling in non-immunized mice (up) and LL01+LL02 group (down). NI: non-immunized.

3.3.3. A single engineered L. lactis strain producing c-di-AMP antigen and TScf adjuvant: a vaccine prototype.

Aiming to the development of a fully integrated mucosal vaccine prototype, a single vector carrying both genes encoding the TScf antigen and the CdaA enzyme responsible for c-di-AMP production was designed. The first attempt to reach this consisted on amplifying from pIQ101 plasmid a DNA region containing the Pcit promoter, the *cdaA* gene and its terminator. After several unsuccessful attempts of cloning this fragment in the BglII restriction site upstream the Pnis promoter in vector pNZTS, the strategy was changed. Instead, TScf coding gene was amplified including the Pnis promoter and terminator of pNZTS plasmid and subcloned in the SalI-PstI restriction sites of vector pIQ101. The resulting p10TS plasmid

containing both genes was finally electroporated in *L. lactis clpP-htrA* strain, and the resulting strain was named *L. lactis* LL10 (Fig 31 and Table XV).

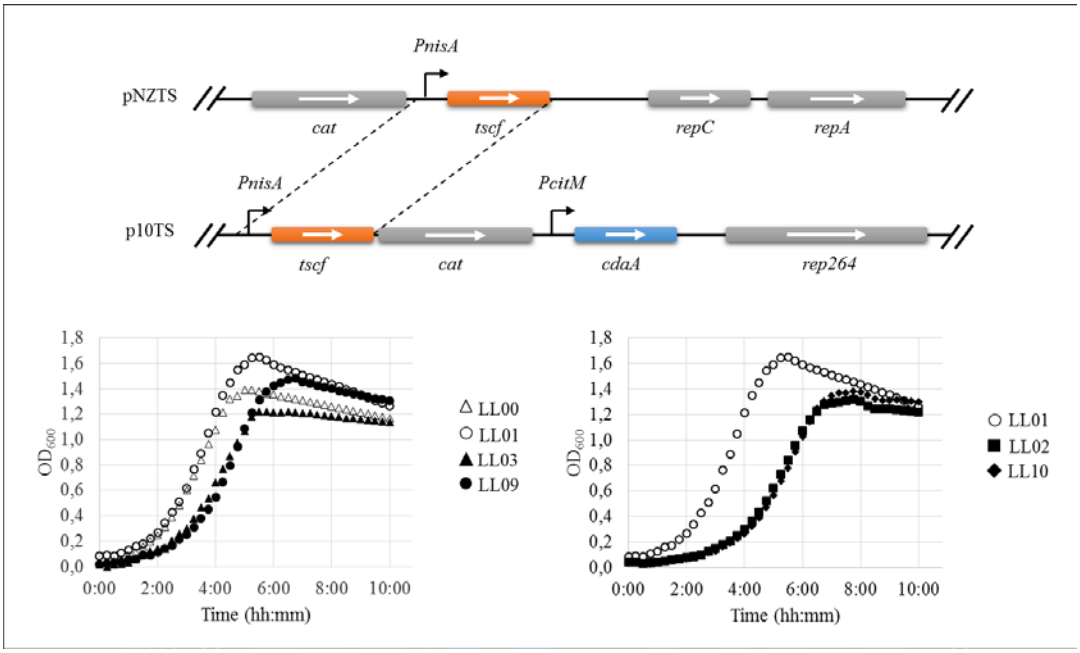

Fig 31 *L. lactis* LL10 strain construction and growth response. Up: cloning scheme for p10TS obtaining. This plasmid contains *tscf* gene under *PnisA* control and *cdaA* gene under *PcitM* control. Down: Growth curves performed in rich medium M17G. LL00: *L. lactis* IL1403 + pBV153, LL01: *L. lactis* NZ9000 *clpP-htrA* + pNZ8048, LL02: *L. lactis* NZ9000 *clpP-htrA* + pNZTS, LL03: *L. lactis* IL1403 + pIQ101, LL09: *L. lactis* NZ9000 *clpP-htrA* + pIQ101, LL10: *L. lactis* NZ9000 *clpP-htrA* + p10TS

In order to standardize conditions for the following immunological experiments, plasmid pIQ101 harboring *cdaA* gene was electroporated in strain *L. lactis clpP-htrA*, originating strain LL09. Since the main target of this chapter is the formulation of a live vaccine, comparative growth curves were performed to check response of *L. lactis* IL1403 and *L. lactis clpP-htrA* strains harboring a trans copy of *cdaA* (Fig 31, down – left). On the other hand the same comparison was done for *L. lactis clpP-htrA* derivative strains harboring *tscf* gene (LL09) and both *tscf* and *cdaA* genes (LL10). Fig 31 shows similar responses in all cases, with homologous behavior for *L. lactis* IL1403 and *L. lactis clpP-htrA* derivative strains.

Moreover, and due to the presence of two different induction systems in strain LL10, an expression test was performed. In the previous chapter, the highest intracellular concentration of c-di-AMP was obtained at pH_0 = 5.5, and cultures were grown until OD_{600} reached 0.50. Since expression of *tscf* was induced once OD_{600} = 0.50 and cultures were then incubated for 3 more hours, the following step was to set up effective conditions for both systems. Therefore, nisin induction for strain LL10 was tested at two time points: simultaneously with inoculation

(OD = 0.05) and at OD_{600} = 0.30, until a final OD_{600} of 0.50 for both cases. Three nisin concentrations were also evaluated; 5, 15 and 50 ng/ml, and strain LL02 induced with 0.5 ng/ml was used as control, for this was the condition in which TScf production was obtained in section 3.3.1.

As seen in Fig 32, the highest overproduction of TScf was evidenced in the crude extract as well as the supernatant fractions when 15 ng/ml nisin was added to growth media at the moment of inoculation (OD_{600} = 0.05).

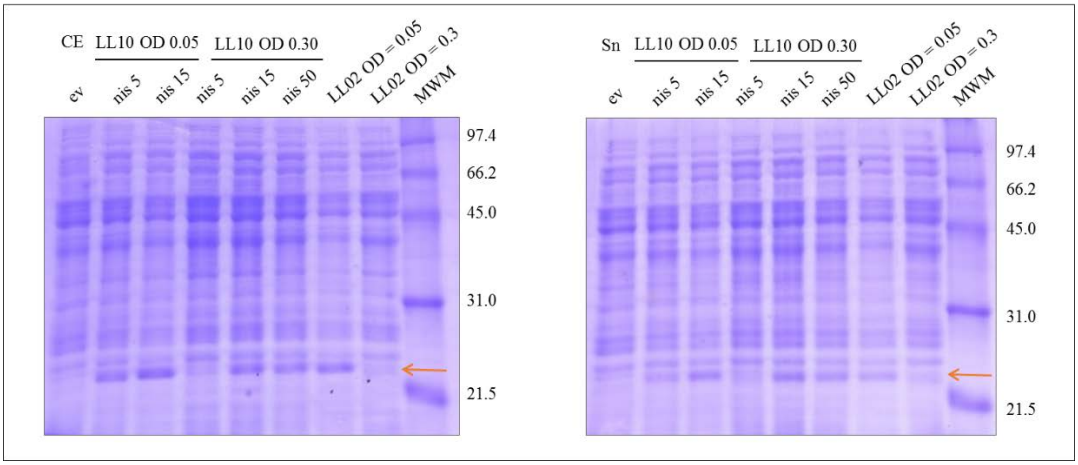

Fig 32 Synthesis of TScf in *L. lactis* LL10. Cultures of *L. lactis* LL10 were grown in rich M17G media supplemented with different nisin concentrations and at different times. Cells were harvested at OD_{600} = 0.5 in all cases. Crude extract (CE, left) and supernatant (Sn, right) fractions evidence a greater expression when 15 ng/ml nisin were added simultaneously with media inoculation. Orange arrows indicate TScf bands. ev: empty vector pNZ8048, MWM: molecular weight marker.

In conclusion, for the immunological experiments performed in the following section inocula were prepared as follows: 2 liters of fresh M17G medium supplemented with the corresponding antibiotics and 15 ng/ml nisin were inoculated with the desired strains at OD_{600} = 0.05 and incubated at 30 ºC until OD_{600} reached 0.5. Cultures were then harvested and washed with PBS, to be finally resuspended at final OD_{600} of 2-3.10^9 CFU/100 µl. An expression check to corroborate TScf presence was finally performed and aliquots were stored at -80 ºC until used (see Materials and Methods).

3.3.4. Immune response of engineered *L. lactis* co-expressing antigen and adjuvant after mucosal administration

With a similar protocol to the one used in section 3.3.2., BALB/C mice were inoculated to analyze the *in vivo* cell-mediated immune response of strain LL10 developed in the previous section. This time inoculated groups were: Non-immunized (NI, mice receiving only PBS); LL10, mice immunized with *L. lactis* LL10 (co-producing TScf and CdaA), LL02, a group of mice receiving bacteria producing the TScf antigen alone (*L. lactis* LL02) and LL09, mice immunized only with the c-di-AMP producer strain (*L. lactis* LL09). A group of mice immunized with *L. lactis* LL01 strain harboring the empty pNZ8048 plasmid and a group receiving TS plus the ISCOMATRIX adjuvant were included as control.

A scheme of three doses separated by 15 days intervals, similar to the one described in the previous section was again used (Fig. 30, A) and footpad swelling was determined 48 hours after the last immunization. In Fig 33 values obtained are plotted for the groups under study, and a clear, significant immune response is obtained for LL10 group. These results evidence that live administration of *L. lactis* LL10 strains elicits a similar response to the control group immunized with purified TS and the ISCOMATRIX adjuvant.

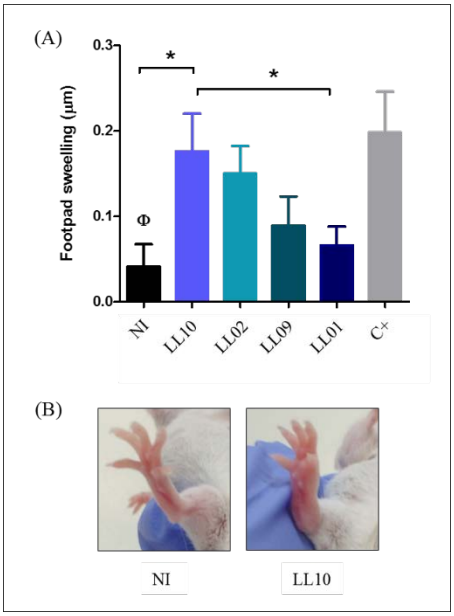

Fig 33 Immune response to *L. lactis* LL10 strain. The immunization protocol as shown in Fig. 30, A was again used to evaluate the response of strain *L. lactis* LL10, co-producing TScf antigen and c-di-AMP adjuvant at the same time. A: Footpad swelling is significantly higher for this group than for NI and control group LL01. B: Footpad swelling developed by LL10 group. NI: Non-immunized.

3.3.5. Conclusions

In this chapter, an engineered *L. lactis* strain was constructed to express *tscf* gene coding a heterologous, immunogenic trans-sialidase (TS) derived peptide (LL02). TScf peptide condenses important T and B epitopes, and Western blot analyses confirmed its production. Furthermore, mice oral inoculation with a combination of strains LL02 and LL09 (derived from strain LL03, obtained in the previous chapter) elicited a clear immune response. This encouraging results lead to the development of strain *L. lactis* LL10, which co-produces in the cytosol the c-di-AMP adjuvant and the optimized TScf antigen.

BALB/c mice oral inoculation with three doses of *L. lactis* LL10, triggered an immune response evidenced by footpad swelling. A diagram of the LL10-based vaccine explaining the combination of the two expression systems employed is presented in Fig 34. These results show that the engineered strain LL10 could result in an effective vaccine for Chagas disease, which is a serious public health problem in South America.

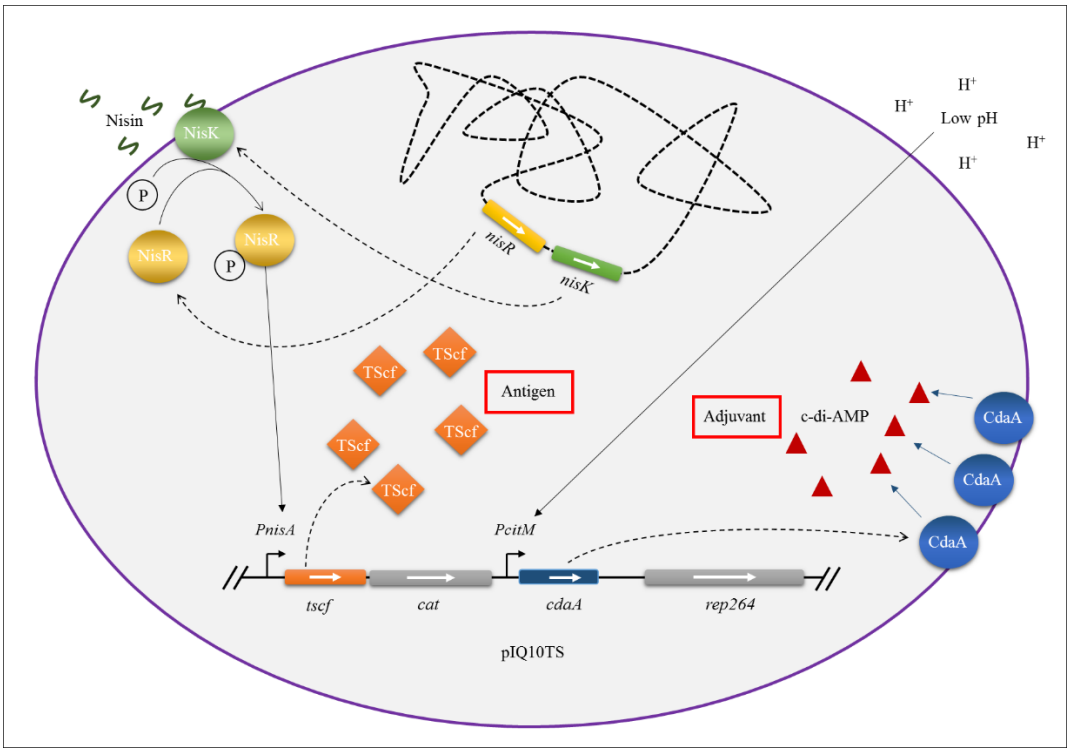

Fig 34 Strain *L. lactis* LL10: an oral vaccine prototype. Presence of *nisRK* two-component system in *L. lactis* genome allows nisin sensing via NisK, upon which NisR is phosphorylated and activates PnisA promoter in pIQ10TS plasmid. On the other hand, low pH activates in turn PcitM promoter, regulating *cdaA*. CdaA is thus synthesized and c-di-AMP produced. Consequently, both TScf antigen and the adjuvant c-di-AMP are co-produced in the cytosol.

3.4. An approach into c-di-AMP metabolism in *E. faecalis* JH2-2

3.4.1. Initial studies into *cdaA* metabolism

As in most low GC firmicutes, only one c-di-AMP synthesizing enzyme is found in *E. faecalis* JH2-2 genome. Being a *cdaA* homologue, it is encoded in the *cdaARglmM* operon, as expected. Interestingly, *glmM* gene in the laboratory model strain *E. faecalis* JH2-2 used in this work, encodes a full 1356 bp open reading frame, whereas the vancomycin-resistant strain V583, presents a shorter version of 1026 bp in its genome, which would result in a 342 amino acids peptide (being the wild type version of 452 amino acids) (Fig 35).

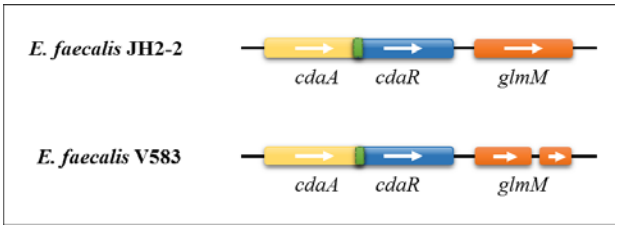

Fig 35 *cdaARglmM* operon in *E. faecalis*. Up: JH2-2 strain shows full version genes (up) whereas in strain V583 (down) a premature stop codon occurs in *glmM* gene.

Moreover, a search for genes present in *E. faecalis* genome coding for proteins with high homology for c-di-AMP degrading enzymes, revealed the presence of a *gdpP* as well as a *pgpH* homologue (Fig 36). Interestingly, the cytosolic version of the DHH family of phosphodiesterases was also found encoded in *E. faecalis* JH2-2 genome. As previously mentioned, the NrnA homologue in *B. subtilis* is not capable of degrading c-di-AMP, but the corresponding protein named Pde2 in *S. pneumoniae* possesses c-di-AMP phosphodiesterase activity, and it was proved that Pde1 (a GdpP homologue) as well as Pde2 affect bacterial growth and virulence (68). In conclusion, *E. faecalis* could count on three c-di-AMP degrading enzymes, and c-di-AMP metabolism in this species could be also related to virulence.

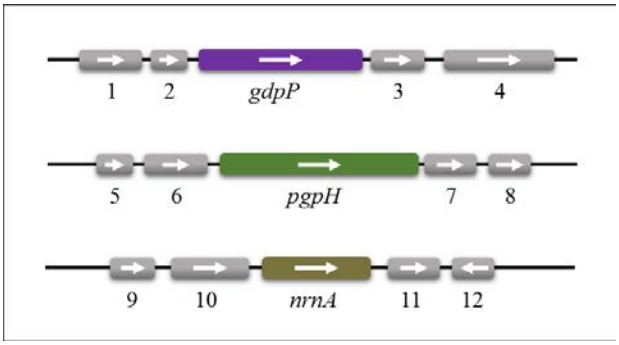

Fig 36 Phosphodiesterases encoded in *E. faecalis* JH2-2 genome. 1: DNA binding protein. 2: SSU ribosomal protein S18p. 3: LSU ribosomal protein L9p. 4: *dnaB*. 5: *gatB*. 6: *phoH*. 7: metal-dependent hydrolase *ybeY*. 8: diacylglycerol kinase. 9: metal-dependent hydrolase. 10: CBS containing protein. 11: *phnA*. 12: hypothetical protein

In the first chapter of this thesis, *L. lactis* Kup proteins were identified as c-di-AMP interaction partners. A search for genes coding for Kup homologues was therefore performed in *E. faecalis* JH2-2, which revealed one copy of *kup* in its genome (Fig 37). Moreover, the presence of other potassium transporters was as well considered in this species. Neither Kdp, nor Ktr homologues were found, but two genes coding for putative members of the Trk potassium transporter family were found. These proteins are predicted as TrkA and TrkH and their genetic contexts are detailed in Fig 37. Interestingly, a homologue to KimA, a novel potassium transporter recently described, was also found encoded in *E. faecalis* JH2-2 genome (49).

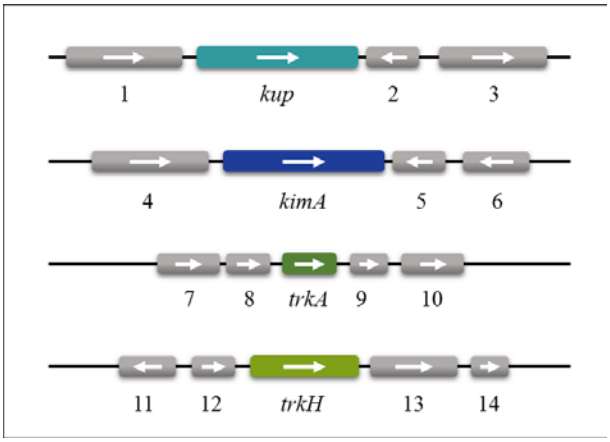

Fig 37 Putative potassium transporters in *E. faecalis* JH2-2. 1: cation-transporting ATPase. 2: transcriptional regulator. 3: Pb, Cd, Zn and Hg transporting ATPase. 4: Co-Zn-Cd resistance protein. 5: acetyl-transferase. 6: L-carnitine choline ABC transporter. 7: *liaS*. 8: *liaR*. 9: hypothetical protein. 10: bactoprenol glucosyl transferase. 11: transcriptional regulator. 12: dihydrofolate reductase. 13: Na^+/H^+ antiporter. 14: transcriptional regulator.

At present, no studies about *E. faecalis* JH2-2 potassium transporters are available. Hence, *kup* gene was cloned in vector pWH844, similarly to *kupA* and *kupB* of *L. lactis* IL1403 in chapter 1. The resulting pIQ311 plasmid was electroporated in *E. coli* LB650 and strain *E. coli* LB13 thus obtained was grown on rich LBG medium to check if a *trans* complementation with *kup* gene could restore growth at low potassium concentrations.

As seen in Fig 38, *E. coli* LB13 strain restores growth on LBG supplemented with 10 mM KCl under induced conditions. Moreover, this strain behaved similarly to LB09-LB11 strains, harboring *trans* copies of high affinity potassium uptake systems. This suggests that *E. faecalis*, similarly to *L. lactis*, counts on the potassium transporter Kup as a high affinity K^+ intake system. However, whether this protein, as well as the rest of the putative potassium transporters previously presented interact with c-di-AMP, remains unknown and proposes interesting future investigations.

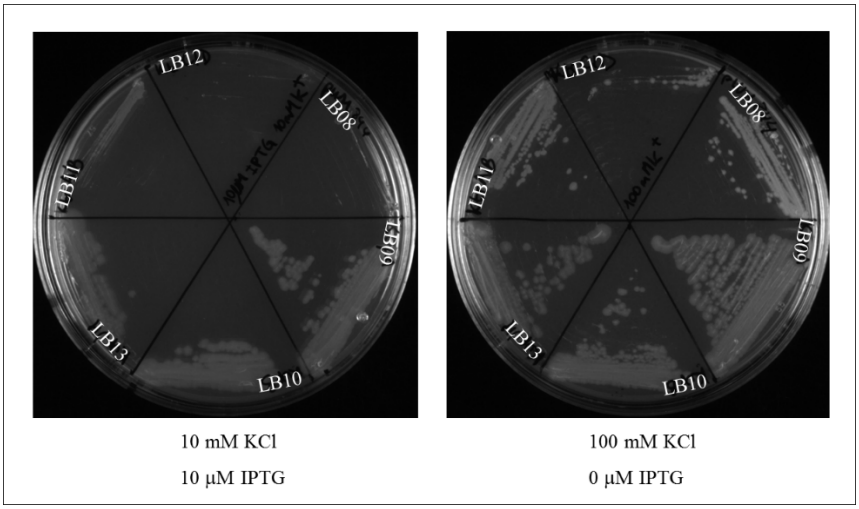

Fig 38 Growth of *E. coli* LB derived strains on LB. LB09 – LB13: *E coli* LB650 complemented with a trans copy of *kupA*, *kupB*, *ktrA/B*, *ktrC/D*, *kup*, respectively. LB08 contains plasmid pWH844, used as negative control. Strains were plated on LB-agar plates supplemented with 10 mM KCl and 10 µM IPTG (left) and 100 mM KCl and no IPTG (right). Low affinity potassium system KtrC/D as well as the negative control with the empty vector only grow at high K^+ concentrations. Expression of *kup* genes and *ktrA/B* system restore growth under low concentrations of KCl.

3.4.2. *ΔgdpP* mutant in *E. faecalis* JH2-2

In order to study the response of *ΔgdpP* mutation in *E. faecalis*, deletion by use of the homologous recombination system previously described in section 3.2.1. and Materials and Methods, was performed. Briefly, up and downstream regions of *gdpP* gene were cloned in the thermosensitive vector pBVGh, and after a series of incubations at the permissive and non-permissive temperatures, a clean deletion occurs. Antibiotic sensitive colonies are isolated, and mutation is checked via internal and external PCR, as well as sequencing (Fig 15).

After confirming strain *E. faecalis* JH12 as a *ΔgdpP* mutant, a phenotypic analysis was carried out on growth curves performed in rich LBG media, supplemented with different stress factors. As seen in Fig 39, this strain does not show a particular phenotype with respect to JH2-2 wild type strain when grown in LBG. Nevertheless, in presence of 5.0 µg/ml ampicillin, a hypersentivity phenotype is evidenced. This response was expected, since similar results were obtained for *L. lactis ΔgdpP* mutants.

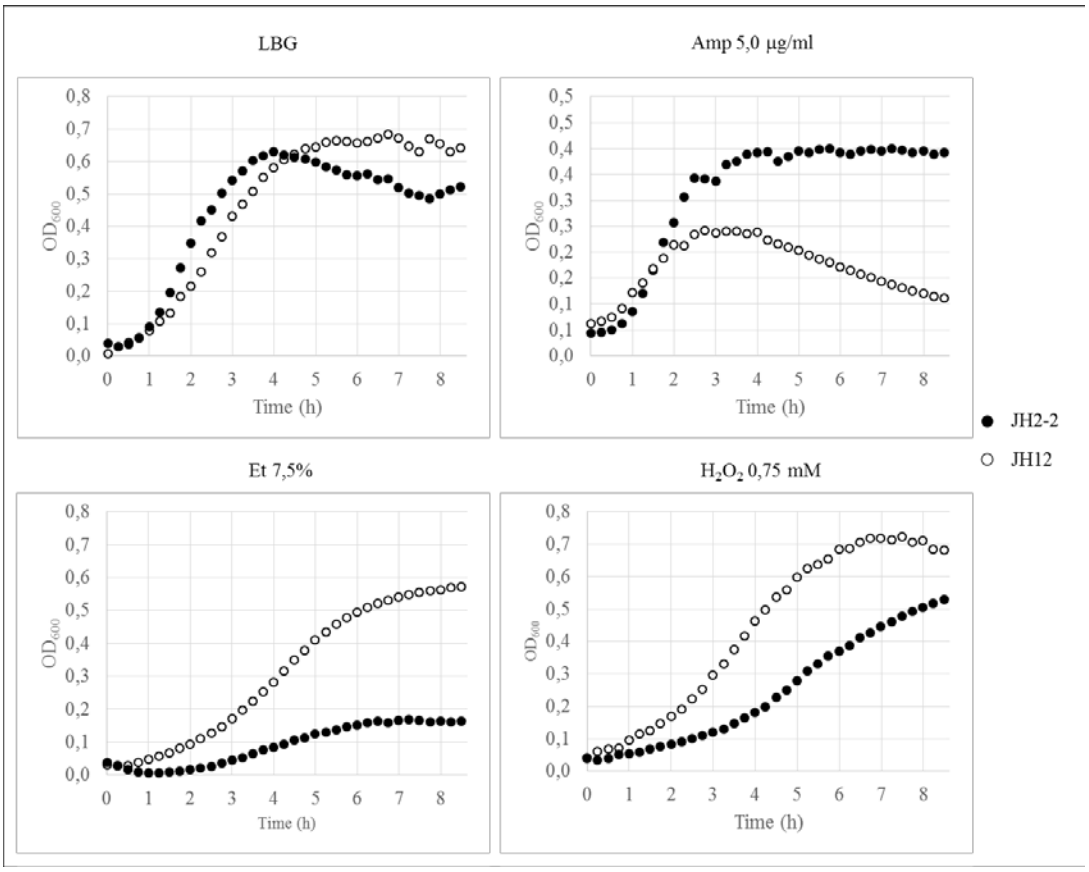

Fig 39 *E. faecalis* JH12 growth in presence of stress factors. Growth curves were performed in rich LBG medium in a microplate reader. Supplements added are indicated above each graph. Similar growth is observed under control LBG conditions. Hypersensitivity phenotype is obtained in presence of 5 µg/ml ampicillin, whereas improved growth was manifested when 7.5% ethanol and 0.75 mM H_2O_2 were added to media. Wild type strain *E. faecalis* JH2-2 was used as control.

Interestingly, a growth advantage is observed for the mutant strain JH12 when LBG is supplemented with 7.5% ethanol and 0.75 mM hydrogen peroxide. On the other hand, even though in LB no clear phenotype was observed when 300 mM KCl was added (not shown), in M17G there was a marked difference, since a hypersensitive phenotype is observed in these conditions (Fig 40). Noteworthy, in M17G control condition, strain JH12 evidences impair growth.

In conclusion, evidence here presented, confirms that in *E. faecalis* c-di-AMP metabolism affects bacterial response to different stress factors. Altered c-di-AMP levels can confer a growth advantage, but it can also be deleterious for the cell. Due to the diversity of pathways influenced by this second messenger, effects on growth curves experiments are hard to analyze and more specific studies are needed. Even in rich control media LBG and M17G, different phenotypes were observed: the lack of GdpP seems not to affect growth in the former, whereas it results deleterious in the latter.

Lastly, whether Kup, as well as the rest of putative potassium transporters previously presented, interact to c-di-AMP remains unknown and proposes interesting future investigations.

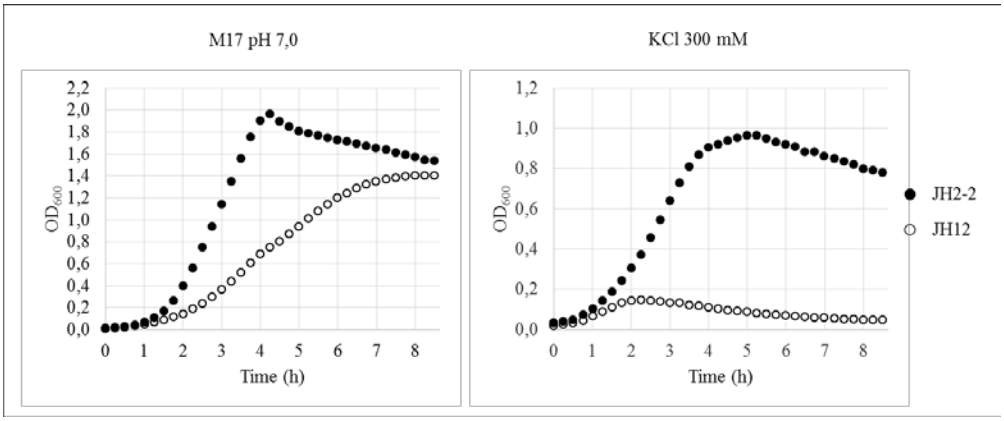

Fig 40 Saline sensitivity in M17G. Growth curves were performed in rich M17G medium in a microplate reader. Addition of 300 mM KCl results in impaired growth for *E. faecalis* JH12 (right).

3.4.3. Virulence analysis of *gdpP* mutant strain *E. faecalis* JH2-2 using the greater wax moth *Galleria mellonella*.

Historically, the use of invertebrate animals, like the silkworm *Bombyx mori* and the fruit fly *Drosophila melanogaster*, has been a very important tool for the development of model microorganisms enabling the study of pathogen-host interactions (179, 180). For instance, the development of the greater wax moth *Galleria mellonella* as an infection model dates from 1938 (181), and due to its adaptability and high proliferation it is a very suitable tool to establish a first approach into the study of pathogenesis and virulence factors (182, 183).

Among the most important advantages for the use of *G. mellonella* larvae, easy manipulation, low cost of colony maintenance and its accepted ethical use can be mentioned. It is also of great relevance the fact that these larvae can be grown at 37ºC, temperature at which human pathogens are adapted and therefore synthesis of most virulence factors occur (184). Moreover, and unlike nematode models, which lack phagocytic immune response (185), the insect *G. mellonella* is capable of eliciting humoral and cellular immune responses. Altogether, *G. mellonella* is a suitable infection model for the analysis of bacterial virulence factors affecting humans and other mammals (186).

E. faecalis JH12 strain was therefore used to inoculate *G. mellonella* larvae and analyze the response and possible phenotypic differences due to the lack of the c-di-AMP degrading enzyme, GdpP (see Materials and Methods). Strain JH13 complemented with a *trans* copy of wild type *gdpP* gene cloned in vector pBV153 was use to check if, in case of obtaining a distinct phenotype for JH12 mutant strain, complementation occurred.

Fig 41 shows survival curves obtained after inoculations with 9.10^6 CFU per larvae. Length of horizontal lines along the x-axis corresponds to serial time, or survival duration until a larva dies. Vertical distances between horizontal lines show accumulated probability changes. Censured subjects are shown as black dots.

In the case under study, the first dead individual occurs at approximately 20 hours after inoculation, in all three cases. Nevertheless, at t = 40 hours, 50% of larvae still survive for the complemented strain JH13, whereas in JH12 mutant and JH2-2 wild type strains the majority of larvae are already dead. Also, Kaplan-Meier survival analysis indicates a significant differences between the three curves, for which it can be said that the absence of *gdpP* gene diminishes virulence in *E. faecalis*, and complementation with vector pIQ104, harboring a wild type copy of *gdpP*, results in an even less virulent strain. Within 50 hours, JH2-2 and JH12 groups are reduced by 90 and 80%, respectively, while 50% of JH13 group remain alive.

A negative control was inoculated with PBS solution, which is completely innocuous (not shown).

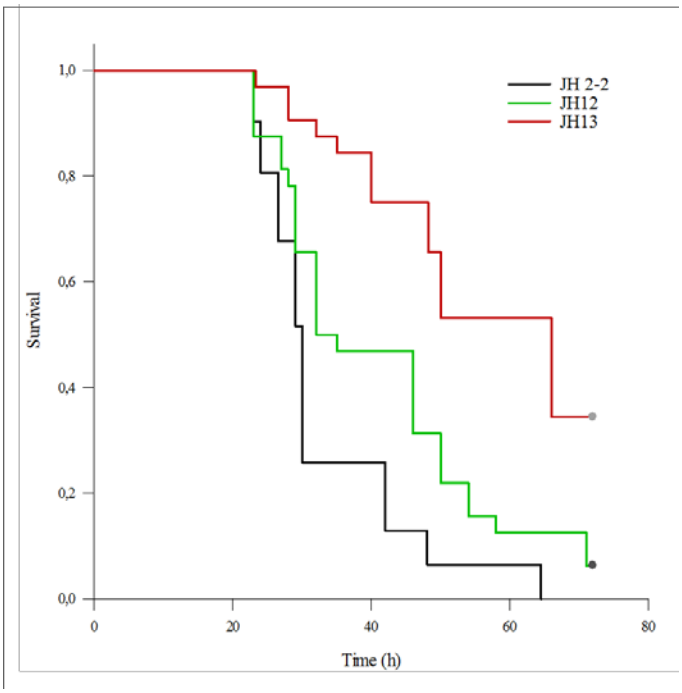

Fig 41 Survival Kaplan-Meier curves for *E. faecalis* strains under study. *G. mellonella* larvae were inoculated with 9.10^6 CFU/larvae. *E. faecalis* JH2-2 (black) was used as control. Green: *ΔgdpP* mutant strain E. faecalis JH12. Red: E. faecalis JH13 (*ΔgdpP* complemented in *trans* with a wild type copy of *gdpP* gene).

3.4.4. Conclusions and Discussion

In this last chapter, an approach was made into c-di-AMP metabolism in *E. faecalis* JH2-2. A bioinformatic analysis of genes related to this second messenger shows that *glmM* could be interrupted in some species of the genus, just as *cdaR* is in *L. lactis* (see below).

Furthermore, putative potassium transporters were found encoded in *E. faecalis* genome. The product of one of them, Kup, was identified as a potassium transporter, and it is likely to have high affinity for this cation like KupA and KupB proteins from *L. lactis*, previously described.

On the other hand, the analysis of *ΔgdpP* on growth curves performed in rich media showed once more for this kind of mutant, a higher susceptibility to ampicillin in LBG and to KCl in M17G. Noteworthy, growth advantage phenotypes were evidenced in presence of ethanol and H_2O_2. Even though a complemented strain was constructed, wild type phenotypes restoration was not achieved (not shown).

Finally, assays in *G. mellonella* showed lower virulence for *ΔgdpP* mutant strain JH12, and even though complemented strain JH13 did not restore wild type phenotype in these experiments either, an even lower virulence was observed. Considering that, as previously mentioned, c-di-AMP levels are tightly regulated, this could be due to the overexpression of *gdpP* resulting in c-di-AMP concentrations below wild type values. Nevertheless, a clear link between c-di-AMP metabolism and virulence was established for *E. faecalis* JH2-2.

4. General Discussions

"Science makes people reach selflessly for truth and objectivity; it teaches people to accept reality, with wonder and admiration, not to mention the deep awe and joy that the natural order of things brings to the true scientist" Lise Meitner

4.1. Novel potassium transporters in *L. lactis* IL1403

Even though potassium is the most abundant cation in the cell and it is essential for life, no K^+ transporter systems have so far been described for *L. lactis* IL1403. *In silico* research showed different systems encoded in other *L. lactis* strains, such as Kdp and Ktr systems, both well studied in model microorganisms like *B. subtilis* and *L. monocytogenes*. Nevertheless, no *kup* gene was found in their genomes, and the absence of this system is also verified in *S. aureus* and *S. pneumonia*. However, it is widely disseminated in numerous species of *Lactobacillus* and *Enterococcus* and it is also present in a few species of *Streptococcus* and *Staphylococcus*.

Interestingly, in the *L. lactis* group, strains with three full copies of the *kup* gene can be found, although the most represented gene arrangement is the two-copy *kupA kupB* cluster, as for the case of *L. lactis* IL1403 presented in this thesis. Nonetheless, some strains only have one copy of the gene, like MG1363 and its derivative NZ9000, the strain of choice for genetic engineering in *L. lactis*. In these cases, a spontaneous mutation in *kupB* originates a stop codon, confirming that only one copy of the gene would be enough to regulate potassium uptake and satisfy the vital metabolic needs of the cell concerning cell turgor, proton motive force, and others.

In the Gram-negative model bacterium *E. coli*, Trchounian and Kobayashi determined in 1999 that the main function of the only Kup in this bacterium (previously known as TrkD) is to uptake K^+ in hyperosmotic conditions at low pH, being a constitutive, low affinity and low rate system (47). Moreover, it is also the case for Gram – bacteria that different amounts of *kup* gene copies can be found in the same genome. In *Legionella pneumophila*, the causal agent of legionelosis, three genes with high similarity to *E. coli kup* gene were found and named *kupA*, *kupB* and *kupC* (187).

As previously mentioned, topology prediction showed that conserved residues are concentrated at the N-terminal end, which was expected since the cytosolic C-terminal end was proved to be non-essential for potassium translocation, whereas truncation of the protein in the loop between membrane regions 10 and 11 resulted in a non-functional peptide (158). Provided *E. coli* has no c-di-AMP synthesizing enzymes and consequently potassium transporters are not expected to bind the second messenger in this bacterium, it is reasonable to expect that the c-di-AMP binding site in *L. lactis* has been acquired at the C-terminal cytosolic half of the protein.

4.2. An updated overview of c-di-AMP in *L. lactis* IL1403

Even though it is well known that synthesis and degradation of c-di-AMP occurs through specific cyclases and phosphodiesterases (CdaA and GdpP respectively) regulation of CdaA by CdaR is still not exactly understood in *L. lactis*. Zhu et al. have suggested that CdaR could be a negative regulator, due to experiments carried out in *E. coli* with different combinations of the *cdaA* operon fragments, although further investigations are needed to confirm these data (82). In the same work, they report that strain MG1363 has a nucleotide deletion in *cdaR* gene, leading to a premature stop codon, and, consequently, to a shorter version of *cdaR*, which is actually annotated as a pseudogene. The resulting peptide would be 96 amino acids long, considerably shorter than the wild type 320 amino acid protein. They also state that no other *L. lactis* strain shows this mutation.

Nonetheless, it seems to be also the case for *L. lactis* IL1403, where a different mutation is found, resulting in a second shorter version of *cdaR*, which would encode a 194 amino acid peptide. From the currently sequenced and assembled 35 *L. lactis* genomes available in the Genbank database, no other *L. lactis* strain shows this unusual genotype, i.e. excluding strains IL1403, MG1363 and its derivative NZ9000, the remaining 32 have the typical gene arrangement, which is represented in Fig 42 by the one present in SK11 strain.

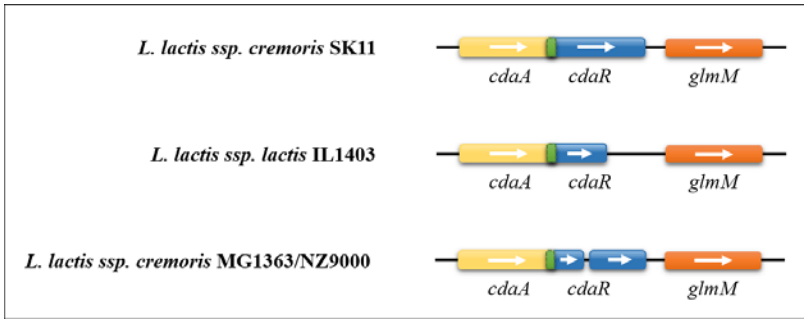

Fig 42 *cdaARglmM* operon in *L. lactis*. Three variations of *cdaR* gene are found among the 35 *L. lactis* sequenced and assembled strains available in the Genbank database.

To discard the possibility that this different version of *cdaR* gene could be due to an annotation error, the whole *cdaARglmM* operon was sequenced, using genomic DNA and primers JN373, JN374, JN385, JN386, JN387, JN388 (see Table XVII). Sequences were assembled and alignment to the theoretical operon available in the Genbank database, allowed confirmation of the premature stop codon.

So far, only one c-di-AMP degrading enzyme was reported in *L. lactis*, GdpP. In this work, levels of c-di-AMP in *ΔgdpP* are maintained close to wild type ones, suggesting that there could be another enzyme in charge of this metabolite hydrolysis. YheB, a *S. pneumonia* Pde2 homologue is therefore proposed, and even though its phosphodiesterase activity was confirmed via the BNPP assay, further more specific studies are needed to confirm if it can degrade c-di-AMP.

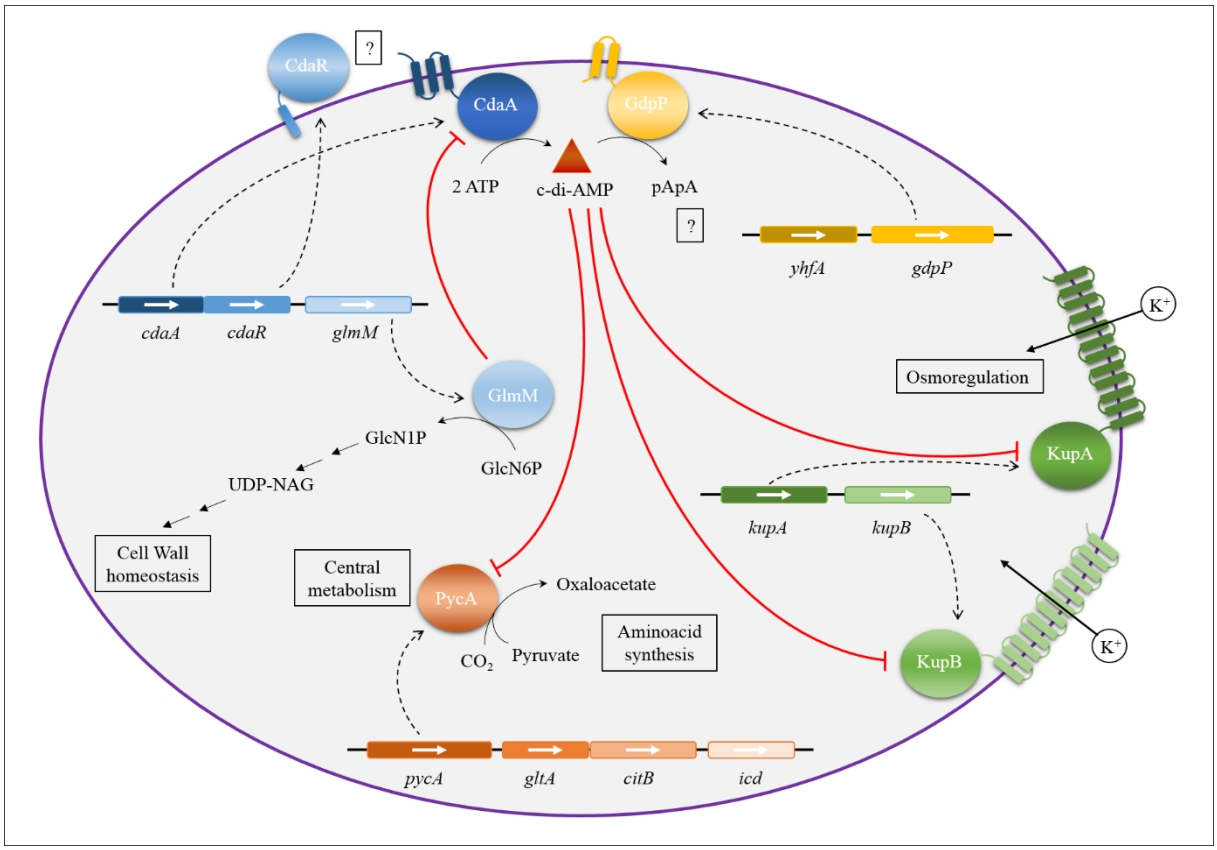

Fig 43. Schematic representation of pathways currently proved to be influenced by c-di-AMP in *L. lactis* IL1403. Red lines represent inhibitory effect. Black curved arrows indicate synthesis of metabolites. Black dashed arrows stand for transcription and translation of indicated genes to obtain the resulting protein. Question marks denote the lack of data to assess the modulation of CdaA by CdaR and of the stringent response metabolite (p)ppGpp by pApA, which has been proved in other model bacteria to modulate the c-di-AMP degrading enzyme GdpP.

Moreover, it is nowadays well known that degradation of c-di-AMP yields phosphoadenylyl phosphoadenosine (pApA) (Fig 43). This metabolite intervenes in turn in the stringent response, a survival pathway activated under carbon source deprivation, although its specific role and its connection to the stringent response metabolite (p)ppGpp which has been proven to modulate GdpP activity in other model organisms (71), remains unknown in *L. lactis*.

The first confirmed modulator of c-di-AMP levels in *L. lactis* IL1403 was GlmM, negatively modulates CdaA. In this way, c-di-AMP metabolism was first linked to cell wall homeostasis in *L. lactis*. Moreover, c-di-AMP was recently proved to be involved in central metabolism in *L. lactis* IL1403 due to the negative regulation it exerts on PycA, the pyruvate carboxylase enzyme in charge of oxaloacetate synthesis (81). This is the first metabolite entering the tricarboxylic acid cycle for CO_2 fixation. Nevertheless, it is important to mention that due to the incomplete Krebs cycle present in *L. lactis*, the main impact of pyruvate carboxylase enzyme is thought to be involved in amino acids synthesis, since oxaloacetate is an aspartate precursor.

Finally, Fig 43 also depicts the novel role of c-di-AMP in *L. lactis* osmoregulation via inhibition of KupA and KupB, identified and described in this thesis. Since other potassium transporters are present in different strains of *L. lactis*, the question remains whether all these systems are also regulated by c-di-AMP, as KupA and KupB were proved to be.

4.3. *L. lactis* + c-di-AMP: a novel vaccine delivery system

Vaccination is one of the most important inventions in the field of public health. As described in the introduction, the first vaccines were obtained based on the use of attenuated or inactivated microorganisms. More recently, molecular techniques opened the possibility to develop vaccines using purified fragments of proteins and recombinant antigens. Even though one of the main advantages of this technology are the fewer risks for the patients, they usually show poor immunogenic properties, making the use of adjuvants necessary to potentiate the specific immune response. In addition, most of the currently used vaccines are administered via parenteral routes, and only a few examples of vaccines administered through mucous membranes are commercially available. Since several pathogens enter their hosts through mucosal surfaces, the development of innovative mucosal vaccines is a challenging paradigm.

L. lactis is a versatile bacterium, which counts on decades of experimental research as well as industrial applications ((188), also developed in the introduction). It is a good candidate when considering the delivery of biologically active immunomodulatory proteins or the production of active biological compounds. Very importantly, the safety of *L. lactis* is well established, having GRAS and QPS status by the Food and Drugs Administration (U.S.A.) and the European Food Safety Authority, respectively. Hence, this microorganism offers a substantial potential as a delivery vector system for vaccines, particularly because it can be

administrated by diverse mucosal routes like oral, nasal or intravaginal, and it survives passage through GIT as well (189).

In this thesis, *L. lactis* LL02 was constructed, allowing overproduction of c-di-AMP more than 15 times above wild type levels. Thus, this tool was combined with the nisin inducible expression system widely study in *L. lactis* to develop strain *L. lactis* LL10. This is a single novel bacterium designed to deliver the TScf antigen adjuvanted by c-di-AMP in the cytosol. Three successive oral immunizations with this prototype of *L. lactis* live vaccine elicited a clear anti-TS cellular response, indicating that oral formulations with this microorganism could be used as delivery of diverse heterologous antigens adjuvanted by c-di-AMP in order to trigger specific immune protection.

The co-existence of both molecules in the same strain of *L. lactis*, not only may favor the development of a specific immune response (by exposing immunocompetent cells to both molecules at the same time), but also may help to reduce the cost vaccination programs in developing countries. Moreover, this prototype *of L. lactis* overproducing c-di-AMP can be combined with other antigens, avoiding expensive and laborious procedures for c-di-AMP production, and enabling systematic research of a variety of antigens.

5. References

"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world" Louis Pasteur

1. Corsetti A, Perpetuini G, Schirone M, Tofalo R, & Suzzi G (2012) Application of starter cultures to table olive fermentation: an overview on the experimental studies. *Frontiers in Microbiology* 3:248.

2. Seixas FN, Rios EA, Martinez de Oliveira AL, Beloti V, & Poveda JM (2018) Selection of Leuconostoc strains isolated from artisanal Serrano Catarinense cheese for use as adjuncts in cheese manufacture. *J Sci Food Agric* 98(10):3899-3906.

3. Bancalari E, et al. (2017) An integrated strategy to discover *Lactobacillus casei* group strains for their potential use as aromatic starters. *Food Res Int* 100(Pt 1):682-690.

4. Liu W, Pang H, Zhang H, & Cai Y (2014) Biodiversity of Lactic Acid Bacteria. *Lactic Acid Bacteria: Fundamentals and Practice*, eds Zhang H & Cai Y (Springer Netherlands, Dordrecht), pp 103-203.

5. Tagg JR, Dajani AS, & Wannamaker LW (1976) Bacteriocins of gram-positive bacteria. *Bacteriol Rev* 40(3):722-756.

6. Rilla N, Martinez B, Delgado T, & Rodriguez A (2003) Inhibition of *Clostridium tyrobutyricum* in Vidiago cheese by *Lactococcus lactis* ssp. *lactis* IPLA 729, a nisin Z producer. *Int J Food Microbiol* 85(1-2):23-33.

7. De Vuyst L & Vandamme EJ (1994) Nisin, a lantibiotic produced by *Lactococcus lactis* subsp. *lactis*: properties, biosynthesis, fermentation and applications. *Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications*, eds De Vuyst L & Vandamme EJ (Springer US, Boston, MA), pp 151-221.

8. Sanders ME (2008) Probiotics: Definition, sources, selection, and uses. *Clinical Infectious Diseases* 46(Supplement_2):S58-S61.

9. Monedero V, Collado MC, & Rodríguez-Díaz J (2018) Therapeutic opportunities in intestinal microbiota-virus interactions. *Trends in Biotechnology* 36(7):645-648.

10. Lee H & Ko G (2016) Antiviral effect of vitamin A on norovirus infection via modulation of the gut microbiome. *Sci Rep* 6:25835.

11. Techo S, Visessanguan W, Vilaichone RK, & Tanasupawat S (2018) Characterization and antibacterial activity against helicobacter pylori of Lactic Acid Bacteria isolated from thai fermented rice noodle. *Probiotics Antimicrob Proteins*:1-11.

12. Noda M, et al. (2018) The expression of bacteriocin production and self-resistance in *Lactobacillus brevis* 174A is mediated by two regulatory proteins. *Appl Environ Microbiol.* doi:10.1128/AEM.02707-17.

13. Noda M, et al. (2015) Characterization and mutational analysis of a two-polypeptide bacteriocin produced by citrus iyo-derived *Lactobacillus brevis* 174A. *Biol Pharm Bull 38*(12):1902-1909.

14. Leroy F & De Vuyst L (2004) Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology* 15(2):67-78.

15. Bassit N, Boquien CY, Picque D, & Corrieu G (1993) Effect of initial oxygen concentration on diacetyl and acetoin production by *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*. *Appl Environ Microbiol* 59(6):1893-1897.

16. Kelleher P, Bottacini F, Mahony J, Kilcawley KN, & van Sinderen D (2017) Comparative and functional genomics of the *Lactococcus lactis* taxon; insights into evolution and niche adaptation. *BMC Genomics* 18(1):267.

17. Chopin A, Chopin MC, Moillo-Batt A, & Langella P (1984) Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. *Plasmid 11*(3):260-263.

18. Bolotin A, et al. (2001) The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res* 11(5):731-753.

19. Gasson MJ (1983) Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic *streptococci* after protoplast-induced curing. *J Bacteriol* 154(1):1-9.

20. Eichenbaum Z, et al. (1998) Use of the lactococcal nisA promoter to regulate gene expression in gram-positive bacteria: comparison of induction level and promoter strength. *Appl Environ Microbiol* 64(8):2763-2769.

21. Mierau I & Kleerebezem M (2005) 10 years of the nisin-controlled gene expression system (NICE) in Lactococcus lactis. *Appl Microbiol Biotechnol* 68(6):705-717.

22. Linares DM, Kok J, & Poolman B (2010) Genome sequences of *Lactococcus lactis* MG1363 (revised) and NZ9000 and comparative physiological studies. *J Bacteriol* 192(21):5806-5812.

23. Cortes-Perez NG, et al. (2006) Construction and characterization of a *Lactococcus lactis* strain deficient in intracellular ClpP and extracellular HtrA proteases. *Microbiology* 152(Pt 9):2611-2618.

24. Fisher K & Phillips C (2009) The ecology, epidemiology and virulence of Enterococcus. Microbiology 155(Pt 6):1749-1757.

25. Boehm AB & Sassoubre LM (2014) Enterococci as indicators of environmental fecal contamination. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection,* eds Gilmore MS, Clewell DB, Ike Y, & Shankar NBoston. https://www.ncbi.nlm.nih.gov/books/NBK190427/

26. Giraffa G (2002) Enterococci from foods. *FEMS Microbiol Rev* 26(2):163-171.

27. Edmond MB, et al. (1995) Vancomycin-resistant *Enterococcus faecium* bacteremia: risk factors for infection. *Clin Infect Dis* 20(5):1126-1133.

28. Abriouel H, et al. (2008) Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical samples. *Int J Food Microbiol* 123(1-2):38-49.

29. Centeno JA, Menendez S, Hermida M, & Rodriguez-Otero JL (1999) Effects of the addition of Enterococcus faecalis in Cebreiro cheese manufacture. *Int J Food Microbiol* 48(2):97-111.

30. Giraffa G (2003) Functionality of *enterococci* in dairy products. *Int J Food Microbiol* 88(2-3):215-222.

31. Nami Y, et al. (2014) A newly isolated probiotic *Enterococcus faecalis* strain from vagina microbiota enhances apoptosis of human cancer cells. *J Appl Microbiol* 117(2):498-508.

32. Mendoza F, Maqueda M, Galvez A, Martinez-Bueno M, & Valdivia E (1999) Antilisterial activity of peptide AS-48 and study of changes induced in the cell envelope properties of an AS-48-adapted strain of *Listeria monocytogenes*. *Appl Environ Microbiol* 65(2):618-625.

33. Abriouel H, Valdivia E, Galvez A, & Maqueda M (1998) Response of *Salmonella choleraesuis* LT2 spheroplasts and permeabilized cells to the bacteriocin AS-48. *Appl Environ Microbiol* 64(11):4623-4626.

34. Khalkhali S & Mojgani N (2017) Bacteriocinogenic potential and virulence traits of *Enterococcus faecium* and *E. faecalis* isolated from human milk. *Iran J Microbiol* 9(4):224-233.

35. Miskin R, Zamir A, & Elson D (1970) Inactivation and reactivation of ribosomal subunits: the peptidyl transferase activity of the 50 s subunit of *Escherihia coli. J Mol Biol* 54(2):355-378.

36. Zamir A, Miskin R, & Elson D (1971) Inactivation and reactivation of ribosomal subunits: amino acyl-transfer RNA binding activity of the 30 s subunit of *Escherichia coli*. *J Mol Biol* 60(2):347-364.

37. Ukai H, Matsuzawa H, Ito K, Yamada M, & Nishimura A (1998) ftsE(Ts) affects translocation of K+-pump proteins into the cytoplasmic membrane of *Escherichia coli*. J Bacteriol 180(14):3663-3670.

38. Follmann M, et al. (2009) Potassium transport in *Corynebacterium glutamicum* is facilitated by the putative channel protein CglK, which is essential for pH homeostasis and growth at acidic pH. *J Bacteriol* 191(9):2944-2952.

39. Krulwich TA, Sachs G, & Padan E (2011) Molecular aspects of bacterial pH sensing and homeostasis. *Nat Rev Microbiol* 9(5):330-343.

40. Epstein W (2003) The roles and regulation of potassium in bacteria. *Prog Nucleic Acid Res Mol Biol* 75:293-320.

41. Radchenko MV, et al. (2006) Potassium/proton antiport system of *Escherichia coli. J Biol Chem* 281(29):19822-19829.

42. Brown AD (1990) Microbial water stress physiology. Principles and perspectives (John Wiley & Sons, Chichester) p xiii + 313 pp.

43. Roberts MF (2005) Organic compatible solutes of halotolerant and halophilic microorganisms. *Saline Systems* 1:5.

44. Trchounian A & Kobayashi H (2000) K+ uptake by fermenting *Escherichia coli* cells: pH dependent mode of the TrkA system operating. *Biosci Rep* 20(4):277-288.

45. Epstein W (2016) The KdpD Sensor Kinase of *Escherichia coli* Responds to several distinct signals to turn on expression of the Kdp transport system. *J Bacteriol* 198(2):212-220.

46. Ballal A, Basu B, & Apte SK (2007) The Kdp-ATPase system and its regulation. *J Biosci* 32(3):559-568.

47. Trchounian A & Kobayashi H (1999) Kup is the major K+ uptake system in *Escherichia coli* upon hyper-osmotic stress at a low pH. *FEBS Lett* 447(2-3):144-148.

48. Holtmann G, Bakker EP, Uozumi N, & Bremer E (2003) KtrAB and KtrCD: two K+ uptake systems in *Bacillus subtilis* and their role in adaptation to hypertonicity. *J Bacteriol* 185(4):1289-1298.

49. Gundlach J, et al. (2017) Control of potassium homeostasis is an essential function of the second messenger cyclic di-AMP in *Bacillus subtilis*. *Sci Signal* 10(475).

50. Corrigan RM, et al. (2013) Systematic identification of conserved bacterial c-di-AMP receptor proteins. *Proc Natl Acad Sci* U S A 110(22):9084-9089.

51. Witte G, Hartung S, Buttner K, & Hopfner KP (2008) Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates. *Mol Cell* 30(2):167-178.

52. Witte CE, et al. (2013) Cyclic di-AMP is critical for *Listeria monocytogenes* growth, cell wall homeostasis, and establishment of infection. *MBio* 4(3):e00282-00213.

53. Oppenheimer-Shaanan Y, Wexselblatt E, Katzhendler J, Yavin E, & Ben-Yehuda S (2011) c-di-AMP reports DNA integrity during sporulation in *Bacillus subtilis*. *EMBO Rep* 12(6):594-601.

54. Gundlach J, et al. (2015) An essential poison: synthesis and degradation of cyclic di-AMP in *Bacillus subtilis. J Bacteriol* 197(20):3265-3274.

55. Woodward JJ, Iavarone AT, & Portnoy DA (2010) c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. *Science* 328(5986):1703-1705.

56. Cron LE, et al. (2011) Two DHH subfamily 1 proteins contribute to pneumococcal virulence and confer protection against pneumococcal disease. *Infect Immun* 79(9):3697-3710.

57. Huynh TN, et al. (2015) An HD-domain phosphodiesterase mediates cooperative hydrolysis of c-di-AMP to affect bacterial growth and virulence. *Proc Natl Acad Sci* U S A 112(7):E747-756.

58. Griffiths JM & O'Neill AJ (2012) Loss of function of the gdpP protein leads to joint beta-lactam/glycopeptide tolerance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 56(1):579-581.

59. Luo Y & Helmann JD (2012) Analysis of the role of *Bacillus subtilis* sigma(M) in beta-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Mol Microbiol* 83(3):623-639.

60. Whiteley AT, Pollock AJ, & Portnoy DA (2015) The PAMP c-di-AMP is essential for *Listeria monocytogenes* growth in rich but not minimal media due to a toxic increase in (p)ppGpp. [corrected]. *Cell Host Microbe* 17(6):788-798.

61. Moscoso JA, et al. (2015) Binding of cyclic di-AMP to the *Staphylococcus aureus* sensor kinase KdpD occurs via the universal stress protein domain and downregulates the expression of the Kdp potassium transporter. *J Bacteriol* 198(1):98-110.

62. Zeden MS, et al. (2018) Cyclic di-adenosine monophosphate (c-di-AMP) is required for osmotic regulation in *Staphylococcus aureus* but dispensable for viability in anaerobic conditions. *J Biol Chem* 293(9):3180-3200.

63. Bai Y, et al. (2012) Mycobacterium tuberculosis Rv3586 (DacA) is a diadenylate cyclase that converts ATP or ADP into c-di-AMP. *PLoS One* 7(4):e35206.

64. Corrigan RM & Grundling A (2013) Cyclic di-AMP: another second messenger enters the fray. *Nat Rev Microbiol* 11(8):513-524.

65. Opoku-Temeng C & Sintim HO (2016) Potent inhibition of cyclic diadenylate monophosphate cyclase by the antiparasitic drug, suramin. *Chem Commun* (Camb) 52(19):3754-3757.

66. Taylor BL & Zhulin IB (1999) PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol Mol Biol Rev* 63(2):479-506.

67. Aravind L & Koonin EV (1998) The HD domain defines a new superfamily of metal-dependent phosphohydrolases. *Trends Biochem Sci* 23(12):469-472.

68. Bai Y, et al. (2013) Two DHH subfamily 1 proteins in *Streptococcus pneumoniae* possess cyclic di-AMP phosphodiesterase activity and affect bacterial growth and virulence. *J Bacteriol* 195(22):5123-5132.

69. Ye M, et al. (2014) DhhP, a cyclic di-AMP phosphodiesterase of *Borrelia burgdorferi*, is essential for cell growth and virulence. *Infect Immun* 82(5):1840-1849.

70. Manikandan K, et al. (2014) Two-step synthesis and hydrolysis of cyclic di-AMP in *Mycobacterium tuberculosis*. *PLoS One* 9(1):e86096.

71. Corrigan RM, Bowman L, Willis AR, Kaever V, & Grundling A (2015) Cross-talk between two nucleotide-signaling pathways in *Staphylococcus aureus*. *J Biol Chem* 290(9):5826-5839.

72. Rao F, et al. (2010) YybT is a signaling protein that contains a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain with ATPase activity. *J Biol Chem* 285(1):473-482.

73. Huynh TN & Woodward JJ (2016) Too much of a good thing: regulated depletion of c-di-AMP in the bacterial cytoplasm. *Curr Opin Microbiol* 30:22-29.

74. Smith WM, et al. (2012) Heat resistance and salt hypersensitivity in *Lactococcus lactis* due to spontaneous mutation of llmg_1816 (*gdpP*) induced by high-temperature growth. *Appl Environ Microbiol* 78(21):7753-7759.

75. Corrigan RM, Abbott JC, Burhenne H, Kaever V, & Grundling A (2011) c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. *PLoS Pathog* 7(9):e1002217.

76. Zhang L, Li W, & He ZG (2013) DarR, a TetR-like transcriptional factor, is a cyclic di-AMP-responsive repressor in *Mycobacterium smegmatis*. *J Biol Chem* 288(5):3085-3096.

77. Bai Y, et al. (2014) Cyclic di-AMP impairs potassium uptake mediated by a cyclic di-AMP binding protein in *Streptococcus pneumoniae*. *J Bacteriol* 196(3):614-623.

78. Chin KH, et al. (2015) Structural insights into the distinct binding mode of cyclic di-AMP with SaCpaA_RCK. *Biochemistry* 54(31):4936-4951.

79. Muller M, Hopfner KP, & Witte G (2015) c-di-AMP recognition by *Staphylococcus aureus PstA*. *FEBS Lett* 589(1):45-51.

80. Schuster CF, et al. (2016) The second messenger c-di-AMP inhibits the osmolyte uptake system OpuC in *Staphylococcus aureus*. *Sci Signal* 9(441):ra81.

81. Sureka K, et al. (2014) The cyclic dinucleotide c-di-AMP is an allosteric regulator of metabolic enzyme function. *Cell* 158(6):1389-1401.

82. Zhu Y, et al. (2016) Cyclic-di-AMP synthesis by the diadenylate cyclase CdaA is modulated by the peptidoglycan biosynthesis enzyme GlmM in *Lactococcus lactis*. *Mol Microbiol* 99(6):1015-1027.

83. Rallu F, Gruss A, Ehrlich SD, & Maguin E (2000) Acid- and multistress-resistant mutants of *Lactococcus lactis*: identification of intracellular stress signals. *Mol Microbiol* 35(3):517-528.

84. Rao F, Ji Q, Soehano I, & Liang ZX (2011) Unusual heme-binding PAS domain from YybT family proteins. *J Bacteriol* 193(7):1543-1551.

85. Tan E, et al. (2013) Solution structure of the PAS domain of a thermophilic YybT protein homolog reveals a potential ligand-binding site. *J Biol Chem* 288(17):11949-11959.

86. Choi PH, et al. (2017) Structural and functional studies of pyruvate carboxylase regulation by cyclic di-AMP in lactic acid bacteria. *Proc Natl Acad Sci* U S A 114(35):E7226-E7235.

87. Kamegaya T, Kuroda K, & Hayakawa Y (2011) Identification of a *Streptococcus pyogenes* SF370 gene involved in production of c-di-AMP. *Nagoya J Med Sci* 73(1-2):49-57.

88. Du B, et al. (2014) Functional analysis of c-di-AMP phosphodiesterase, GdpP, in *Streptococcus suis* serotype 2. *Microbiol Res* 169(9-10):749-758.

89. Du B & Sun JH (2015) Diadenylate cyclase evaluation of ssDacA (SSU98_1483) in *Streptococcus suis* serotype 2. *Genet Mol Res* 14(2):6917-6924.

90. Cho KH & Kang SO (2013) *Streptococcus pyogenes* c-di-AMP phosphodiesterase, GdpP, influences SpeB processing and virulence. *PLoS One* 8(7):e69425.

91. Underwood AJ, Zhang Y, Metzger DW, & Bai G (2014) Detection of cyclic di-AMP using a competitive ELISA with a unique pneumococcal cyclic di-AMP binding protein. *J Microbiol Methods* 107:58-62.

92. Cheng X, et al. (2016) Regulation of oxidative response and extracellular polysaccharide synthesis by a diadenylate cyclase in *Streptococcus mutans*. *Environ Microbiol* 18(3):904-922.

93. Peng X, Michalek S, & Wu H (2016) Effects of diadenylate cyclase deficiency on synthesis of extracellular polysaccharide matrix of *Streptococcus mutans* revisit. *Environ Microbiol* 18(11):3612-3619.

94. Peng X, Zhang Y, Bai G, Zhou X, & Wu H (2016) Cyclic di-AMP mediates biofilm formation. *Mol Microbiol* 99(5):945-959.

95. Andrade WA, et al. (2016) Group B Streptococcus degrades cyclic-di-AMP to modulate STING-dependent type I interferon production. *Cell Host Microbe* 20(1):49-59.

96. Wang X, et al. (2017) A novel phosphodiesterase of the GdpP family modulates cyclic di-AMP levels in response to cell membrane stress in daptomycin-resistant *Enterococci*. *Antimicrob Agents Chemother* 61(3).

97. Sales Junior PA, et al. (2017) Experimental and clinical treatment of chagas disease: a review. *Am J Trop Med Hyg* 97(5):1289-1303.

98. Dias JC (2007) Southern Cone Initiative for the elimination of domestic populations of Triatoma infestans and the interruption of transfusional Chagas disease. Historical aspects, present situation, and perspectives. *Mem Inst Oswaldo Cruz* 102 Suppl 1:11-18.

99. Thakare R, Dasgupta A, & Chopra S (2018) An update on benznidazole for the treatment of patients with Chagas disease. *Drugs Today* (Barc) 54(1):15-23.

100. Olivera MJ, Fory JA, & Olivera AJ (2017) Therapeutic drug monitoring of benznidazole and nifurtimox: a systematic review and quality assessment of published clinical practice guidelines. *Rev Soc Bras Med Trop* 50(6):748-755.

101. Schenkman S, Jiang MS, Hart GW, & Nussenzweig V (1991) A novel cell surface trans-sialidase of *Trypanosoma cruzi* generates a stage-specific epitope required for invasion of mammalian cells. *Cell* 65(7):1117-1125.

102. Ming M, Chuenkova M, Ortega-Barria E, & Pereira ME (1993) Mediation of *Trypanosoma cruzi* invasion by sialic acid on the host cell and trans-sialidase on the trypanosome. *Mol Biochem Parasitol* 59(2):243-252.

103. Villalta F, Smith CM, Ruiz-Ruano A, & Lima MF (2001) A ligand that *Trypanosoma cruzi* uses to bind to mammalian cells to initiate infection. *FEBS Lett* 505(3):383-388.

104. Schenkman S, Eichinger D, Pereira ME, & Nussenzweig V (1994) Structural and functional properties of *Trypanosoma* trans-sialidase. *Annu Rev Microbiol* 48:499-523.

105. Dumonteil E, Escobedo-Ortegon J, Reyes-Rodriguez N, Arjona-Torres A, & Ramirez-Sierra MJ (2004) Immunotherapy of *Trypanosoma cruzi* infection with DNA vaccines in mice. *Infect Immun* 72(1):46-53.

106. Martinez-Campos V, et al. (2015) Expression, purification, immunogenicity, and protective efficacy of a recombinant Tc24 antigen as a vaccine against *Trypanosoma cruzi* infection in mice. *Vaccine* 33(36):4505-4512.

107. Villanueva-Lizama LE, et al. (2018) *Trypanosoma cruzi* vaccine candidate antigens Tc24 and TSA-1 recall memory immune response associated with HLA-A and -B supertypes in Chagasic chronic patients from Mexico. *PLoS Negl Trop Dis* 12(1):e0006240.

108. Riedel S (2005) Edward Jenner and the history of smallpox and vaccination. *Proc* (Bayl Univ Med Cent) 18(1):21-25.

109. Lopez AL, Gonzales MLA, Aldaba JG, & Nair GB (2014) Killed oral cholera vaccines: history, development and implementation challenges. *Therapeutic Advances in Vaccines* 2(5):123-136.

110. Koury R & Warrington SJ (2017) Rabies. StatPearls, Treasure Island (FL)). https://www.ncbi.nlm.nih.gov/books/NBK448076/

111. Pliaka V, Kyriakopoulou Z, & Markoulatos P (2012) Risks associated with the use of live-attenuated vaccine poliovirus strains and the strategies for control and eradication of paralytic poliomyelitis. *Expert Rev Vaccines* 11(5):609-628.

112. Baxter D (2007) Active and passive immunity, vaccine types, excipients and licensing. *Occupational Medicine* 57(8):552-556.

113. Clark Z & Maurer DM (2018) Pneumococcal vaccines in chronic obstructive pulmonary disease. *Am Fam Physician* 97(7):463-464.

114. Gupta RK & Siber GR (1995) Adjuvants for human vaccines--current status, problems and future prospects. *Vaccine* 13(14):1263-1276.

115. Klijn N, Weerkamp AH, & de Vos WM (1995) Genetic marking of Lactococcus lactis shows its survival in the human gastrointestinal tract. *Appl Environ Microbiol* 61(7):2771-2774.

116. Steidler L, et al. (2000) Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science 289(5483):1352-1355.

117. Iwaki M, et al. (1990) Oral immunization with recombinant Streptococcus lactis carrying the *Streptococcus* mutans surface protein antigen gene. *Infect Immun* 58(9):2929-2934.

118. Braat H, et al. (2006) A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clin Gastroenterol Hepatol* 4(6):754-759.

119. Vandenbroucke K, et al. (2010) Orally administered *L. lactis* secreting an anti-TNF nanobody demonstrate efficacy in chronic colitis. *Mucosal Immunol* 3(1):49-56.

120. Wells JM, Wilson PW, Norton PM, Gasson MJ, & Le Page RW (1993) *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. *Mol Microbiol* 8(6):1155-1162.

121. Saez D, Fernandez P, Rivera A, Andrews E, & Onate A (2012) Oral immunization of mice with recombinant *Lactococcus lactis* expressing Cu,Zn superoxide dismutase of Brucella abortus triggers protective immunity. *Vaccine* 30(7):1283-1290.

122. Asensi GF, et al. (2013) Oral immunization with *Lactococcus lactis* secreting attenuated recombinant staphylococcal enterotoxin B induces a protective immune response in a murine model. *Microb Cell Fact* 12:32.

123. Ahmed B, Loos M, Vanrompay D, & Cox E (2013) Mucosal priming of the murine immune system against enterohemorrhagic Escherichia coli O157:H7 using *Lactococcus lactis* expressing the type III secretion system protein EspB. *Vet Immunol Immunopathol* 152(1-2):141-145.

124. Cauchard S, et al. (2011) Mucosal co-immunization of mice with recombinant lactococci secreting VapA antigen and leptin elicits a protective immune response against *Rhodococcus equi* infection. *Vaccine* 30(1):95-102.

125. Marelli B, Perez AR, Banchio C, de Mendoza D, & Magni C (2011) Oral immunization with live *Lactococcus lactis* expressing rotavirus VP8 subunit induces specific immune response in mice. *J Virol Methods* 175(1):28-37.

126. Bermudez-Humaran LG, et al. (2005) A novel mucosal vaccine based on live *Lactococci* expressing E7 antigen and IL-12 induces systemic and mucosal immune responses and protects mice against human papillomavirus type 16-induced tumors. *J Immunol* 175(11):7297-7302.

127. Cano-Garrido O, Seras-Franzoso J, & Garcia-Fruitos E (2015) Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes. *Microb* 1.

Lasztity R (2009) Food Quality And Standards - Volume III (EOLSS Publishers Company Limited).

128. Pashine A, Valiante NM, & Ulmer JB (2005) Targeting the innate immune response with improved vaccine adjuvants. *Nat Med* 11(4 Suppl):S63-68.

129. Apost, et al. (2016) Adjuvants: classification, modus operandi, and licensing. journal of immunology *Research* 2016:16.

130. Ebensen T, et al. (2011) Bis-(3',5')-cyclic dimeric adenosine monophosphate: strong Th1/Th2/Th17 promoting mucosal adjuvant. *Vaccine* 29(32):5210-5220.

131. Rueckert C, et al. (2017) Cyclic dinucleotides modulate induced type I IFN responses in innate immune cells by degradation of STING. *FASEB J* 31(7):3107-3115.

132. Matos MN, et al. (2017) Immunization with Tc52 or its amino terminal domain adjuvanted with c-di-AMP induces Th17+Th1 specific immune responses and confers protection against *Trypanosoma cruzi*. *PLoS Negl Trop Dis* 11(2):e0005300.

133. Landi A, et al. (2017) Superior immunogenicity of HCV envelope glycoproteins when adjuvanted with cyclic-di-AMP, a STING activator or archaeosomes. *Vaccine* 35(50):6949-6956.

134. Ebensen T, et al. (2017) Mucosal administration of cycle-di-nucleotide-adjuvanted virosomes efficiently induces protection against Influenza H5N1 in mice. *Front Immunol* 8:1223.

135. Weiss W, Weiland F, & Gorg A (2009) Protein detection and quantitation technologies for gel-based proteome analysis. *Methods Mol Biol* 564:59-82.

136. Efron B (1988) Logistic regression, Survival Analysis, and the Kaplan-Meier curve. 83(402):414-425.

137. Bertona D, et al. (2017) Development and assessment of a new cage-like particle adjuvant. J Pharm Pharmacol 69(10):1293-1303.

138. Commichau FM, Gibhardt J, Halbedel S, Gundlach J, & Stulke J (2017) A delicate connection: c-di-AMP affects cell integrity by controlling osmolyte transport. *Trends Microbiol.* VOLUME 26, ISSUE 3, P175-185

139. Commichau FM, Dickmanns A, Gundlach J, Ficner R, & Stulke J (2015) A jack of all trades: the multiple roles of the unique essential second messenger cyclic di-AMP. *Mol Microbiol* 97(2):189-204.

140. Gundlach J, Commichau FM, & Stulke J (2017) Perspective of ions and messengers: an intricate link between potassium, glutamate, and cyclic di-AMP. *Curr Genet.* Volume 64, Issue 1, pp 191–195

141. Quintero FJ & Blatt MR (1997) A new family of K+ transporters from *Arabidopsis* that are conserved across phyla. *FEBS Lett* 415(2):206-211.

142. Sato Y, et al. (2014) Defining membrane spanning domains and crucial membrane-localized acidic amino acid residues for K(+) transport of a Kup/HAK/KT-type *Escherichia coli* potassium transporter. *J Biochem* 155(5):315-323.

143. Jiang Y, Pico A, Cadene M, Chait BT, & MacKinnon R (2001) Structure of the RCK domain from the E. coli K+ channel and demonstration of its presence in the human BK channel. *Neuron* 29(3):593-601.

144. Schlosser A, Meldorf M, Stumpe S, Bakker EP, & Epstein W (1995) TrkH and its homolog, TrkG, determine the specificity and kinetics of cation transport by the Trk system of *Escherichia coli. J Bacteriol* 177(7):1908-1910.

145. Williams T, Bauer S, Beier D, & Kuhn M (2005) Construction and characterization of Listeria monocytogenes mutants with in-frame deletions in the response regulator genes identified in the genome sequence. *Infect Immun* 73(5):3152-3159.

146. Cocaign-Bousquet M, Garrigues C, Novak L, Lindley ND, & Loublere P (1995) Rational development of a simple synthetic medium for the sustained growth of *Lactococcus lactis. Journal of Applied Bacteriology* 79(1):108-116.

147. Zhang G, Mills DA, & Block DE (2009) Development of chemically defined media supporting high-cell-density growth of *lactococci*, *enterococci*, and *streptococci*. *Appl Environ* Microbiol 75(4):1080-1087.

148. Marelli B & Magni C (2009) A simple expression system for *Lactococcus lactis* and *Enterococcus faecalis*. *World J Microbiol Biotechnol* 26(6):999-1007.

149. Blancato VS & Magni C (2010) A chimeric vector for efficient chromosomal modification in *Enterococcus faecalis* and other lactic acid bacteria. *Lett Appl Microbiol* 50(5):542-546.

150. Waxman DJ & Strominger JL (1983) Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics. *Annu Rev Biochem* 52:825-869.

151. Hubschwerlen C (2007) 7.17 - β-Lactam Antibiotics A2 - Taylor, John B. *Comprehensive Medicinal Chemistry II*, ed Triggle DJ (Elsevier, Oxford), pp 479-518.

152. Watanakunakorn C (1984) Mode of action and in-vitro activity of vancomycin. *J Antimicrob Chemother* 14 Suppl D:7-18.

153. Salton MR (1958) The lysis of micro-organisms by lysozyme and related enzymes. *J Gen Microbiol* 18(2):481-490.

154. Rismondo J, et al. (2016) Phenotypes associated with the essential diadenylate cyclase CdaA and its potential regulator CdaR in the human pathogen *Listeria monocytogenes*. *J Bacteriol* 198(3):416-426.

155. Yang J, et al. (2014) Deletion of the cyclic di-AMP phosphodiesterase gene (cnpB) in *Mycobacterium tuberculosis* leads to reduced virulence in a mouse model of infection. *Mol Microbiol* 93(1):65-79.

156. Mehne FM, et al. (2013) Cyclic di-AMP homeostasis in *Bacillus subtilis*: both lack and high level accumulation of the nucleotide are detrimental for cell growth. *J Biol Chem* 288(3):2004-2017.

157. Freire-de-Lima L, Fonseca LM, Oeltmann T, Mendonca-Previato L, & Previato JO (2015) The trans-sialidase, the major *Trypanosoma cruzi* virulence factor: Three decades of studies. *Glycobiology* 25(11):1142-1149.

158. Bontempi IA, et al. (2015) Efficacy of a trans-sialidase-ISCOMATRIX subunit vaccine candidate to protect against experimental Chagas disease. *Vaccine* 33(10):1274-1283.

159. Bontempi I, et al. (2017) Trans-sialidase overcomes many antigens to be used as a vaccine candidate against *Trypanosoma cruzi*. *Immunotherapy* 9(7):555-565.

160. Rosenberg CS, Martin DL, & Tarleton RL (2010) CD8+ T cells specific for immunodominant trans-sialidase epitopes contribute to control of *Trypanosoma cruzi* infection but are not required for resistance. *J Immunol* 185(1):560-568.

161. Eickhoff CS, et al. (2011) Co-administration of a plasmid DNA encoding IL-15 improves long-term protection of a genetic vaccine against *Trypanosoma cruzi*. *PLoS Negl Trop Dis* 5(3):e983.

162. Alvarez P, Leguizamon MS, Buscaglia CA, Pitcovsky TA, & Campetella O (2001) Multiple overlapping epitopes in the repetitive unit of the shed acute-phase antigen from *Trypanosoma cruzi* enhance its immunogenic properties. *Infect Immun* 69(12):7946-7949.

163. Matsumoto Y, Ishii M, Shimizu K, Kawamoto S, & Sekimizu K (2017) A silkworm infection model to evaluate antifungal drugs for Cryptococcosis. *Med Mycol J* 58(4):E131-E137.

164. Mondotte JA & Saleh MC (2018) Antiviral immune response and the route of infection in *Drosophila melanogaster*. *Adv Virus Res* 100:247-278.

165. Smith TL (1938) Genetical Studies on the wax moth *Galleria mellonella* Linn. *Genetics* 23(1):115-137.

166. Rakic Martinez M, Wiedmann M, Ferguson M, & Datta AR (2017) Assessment of Listeria monocytogenes virulence in the *Galleria mellonella* insect larvae model. *PLoS One* 12(9):e0184557.

167. Meir M, Grosfeld T, & Barkan D (2018) Establishment and validation of *Galleria mellonella* as a novel model organism to study *Mycobacterium abscessus* infection, pathogenesis, and treatment. *Antimicrob Agents Chemother* 62(4).

168. Cook SM & McArthur JD (2013) Developing *Galleria mellonella* as a model host for human pathogens. *Virulence* 4(5):350-353.

169. Ermolaeva MA & Schumacher B (2014) Insights from the worm: the *C. elegans* model for innate immunity. *Semin Immunol* 26(4):303-309.

170. Kavanagh K & Reeves EP (2004) Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. *FEMS Microbiol Rev* 28(1):101-112.

171. Hori JI, Pereira MS, Roy CR, Nagai H, & Zamboni DS (2013) Identification and functional characterization of K(+) transporters encoded by *Legionella pneumophila kup* genes. *Cell Microbiol* 15(12):2006-2019.

172. Song AA, In LLA, Lim SHE, & Rahim RA (2017) A review on *Lactococcus lactis*: from food to factory. *Microb Cell Fact* 16(1):55.

173. Wells JM & Mercenier A (2008) Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. *Nat Rev Microbiol* 6(5):349-362.*Cell Fact* 14:137.

174. Hamann, A. 1991. PhD thesis. University of Osnabrück, Osnabrück, Federal Republic of Germany.

175. Studier and Moffatt, 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol.* 1986 May 5;189(1):113-30.

176. Hanahan, 1983. Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol.* 1983 Jun 5;166(4):557-80.

177. Law et al. 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. *J Bacteriol.* 1995 Dec;177(24):7011-8.

178. Guzman et al. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol*. 1995 Jul;177(14):4121-30.

179. De Ruyter et al. 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl Environ Microbiol.* 1996 Oct;62(10):3662-7.

180. Grubek, Z. Ms. Thesis 2016. Georg August Universität, Göttingen, Germany.

6. Appendix

“Valid criticism does you a favor” Carl Sagan

6.1. Peptide identification after c-di-AMP pull-down experiment.

Table XIV Peptide identification after c-di-AMP pull-down experiment

Identified Protein	Accession Number	Molecular Weight	+	-	Ratio +/-
DNA polymerase III, subunits beta and tau	Q9CDM5_LACLA	61 kDa	34	< 0.5	69,00
30S ribosomal protein S6	RS6_LACLA	11 kDa	15	< 0.5	31,00
Uncharacterized protein	Q9CIR6_LACLA	62 kDa	13	< 0.5	27,00
PpGpp synthetase I	Q9CJ94_LACLA	84 kDa	11	< 0.5	23,00
Protease	Q9CE72_LACLA	47 kDa	11	< 0.5	23,00
Uncharacterized protein	Q9CED1_LACLA	12 kDa	11	< 0.5	23,00
3-hydroxy-3-methylglutaryl coenzyme A reductase	Q9CFB1_LACLA	45 kDa	10	< 0.5	21,00
6,7-dimethyl-8-ribityllumazine synthase	RISB_LACLA	17 kDa	10	< 0.5	21,00
Aspartate racemase	Q9CDJ4_LACLA	28 kDa	8	< 0.5	17,00
Uncharacterized protein	Q9CEX0_LACLA	22 kDa	8	< 0.5	17,00
Initiation-control protein YabA	YABA_LACLA	13 kDa	8	< 0.5	17,00
ATP-dependent DNA helicase RecG	Q9CDH6_LACLA	75 kDa	7	< 0.5	15,00
Pyrimidine-nucleoside phosphorylase	Q9CFM5_LACLA	47 kDa	7	< 0.5	15,00
Oxidoreductase	Q9CEV1_LACLA	28 kDa	7	< 0.5	15,00
Uncharacterized protein	Q9CFC1_LACLA	21 kDa	7	< 0.5	15,00
Uncharacterized protein	Q9CDN8_LACLA	17 kDa	7	< 0.5	15,00
Single-stranded DNA specific exonuclease	Q9CHT6_LACLA	84 kDa	6	< 0.5	13,00
Glutamine ABC transporter permease and substrate binding protein	Q9CES5_LACLA	78 kDa	6	< 0.5	13,00
Peptide chain release factor 2	RF2_LACLA	42 kDa	6	< 0.5	13,00
Uncharacterized protein	Q9CHI4_LACLA	10 kDa	6	< 0.5	13,00
Cell-division ATP-binding protein FtsE	Q9CGX0_LACLA	26 kDa	6	< 0.5	13,00
DNA repair protein RecN	Q9CH78_LACLA	63 kDa	34	3	9,86
Uncharacterized protein	Q9CFB8_LACLA	15 kDa	45	5	8,27
Aspartate--tRNA ligase	SYD_LACLA	67 kDa	164	20	8,02
Uncharacterized protein	Q9CEI1_LACLA	13 kDa	26	3	7,57
DNA mismatch repair protein MutL	MUTL_LACLA	74 kDa	30	4	6,78
Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	GLMS_LACLA	66 kDa	206	31	6,56

Phosphoenolpyruvate-protein phosphotransferase	PT1_LACLA	63 kDa	126	19	6,49
Septation ring formation regulator EzrA	EZRA_LACLA	66 kDa	35	5	6,45
NADH oxidase	Q9CIG9_LACLA	49 kDa	38	6	5,92
Elongation factor 4	LEPA_LACLA	68 kDa	32	5	5,91
ABC transporter ATP binding protein	Q9CJA0_LACLA	58 kDa	61	10	5,86
Uncharacterized protein	Q9CGM2_LACLA	14 kDa	14	2	5,80
N-acetylmuramidase	Q9CED5_LACLA	50 kDa	14	2	5,80
Universal stress protein	Q9CEI3_LACLA	16 kDa	120	21	5,60
Arginine--tRNA ligase	SYR_LACLA	63 kDa	30	5	5,55
Folylpolyglutamate synthase	Q9CGE0_LACLA	48 kDa	12	2	5,00
Homoserine dehydrogenase	DHOM_LACLA	47 kDa	21	4	4,78
Uncharacterized protein	Q9CGJ5_LACLA	19 kDa	11	2	4,60
Hydroxymethylglutaryl-CoA synthase	Q9CFA9_LACLA	43 kDa	11	2	4,60
Gamma-glutamyl phosphate reductase	PROA_LACLA	45 kDa	24	5	4,45
Sugar ABC transporter ATP binding protein	Q9CFY3_LACLA	56 kDa	15	3	4,43
ABC transporter ATP binding protein	Q9CFC6_LACLA	70 kDa	31	7	4,20
Glucose-6-phosphate isomerase	G6PI_LACLA	50 kDa	10	2	4,20
Peptide-binding protein	Q9CI27_LACLA	60 kDa	14	3	4,14
Uncharacterized protein	Q9CJ15_LACLA	13 kDa	18	4	4,11
Peptide chain release factor 3	RF3_LACLA	60 kDa	30	7	4,07
ABC transporter ATP binding protein	Q9CJC5_LACLA	61 kDa	62	15	4,03
Chaperone protein DnaK	DNAK_LACLA	65 kDa	150	37	4,01
Lysine--tRNA ligase	SYK_LACLA	57 kDa	21	5	3,91
Probable phosphoketolase	PHK_LACLA	93 kDa	9	2	3,80
Uncharacterized protein	Q9CDZ4_LACLA	18 kDa	43	11	3,78
Uncharacterized protein	Q9CID7_LACLA	17 kDa	68	18	3,70
Peptide chain release factor 1	RF1_LACLA	40 kDa	27	7	3,67
Uncharacterized protein	Q9CFU3_LACLA	15 kDa	16	4	3,67
UPF0145 protein YjfJ	YJFJ_LACLA	12 kDa	30	8	3,59
Signal recognition particle receptor FtsY	Q9CHB9_LACLA	51 kDa	392	109	3,58
Uncharacterized protein	Q9CF71_LACLA	56 kDa	12	3	3,57
Polysaccharide biosynthesis protein	Q9CIZ9_LACLA	70 kDa	12	3	3,57
UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2	MURA2_LACLA	45 kDa	12	3	3,57

Aromatic amino acid specific aminotransferase	Q9CJE0_LACLA	43 kDa	60	17	3,46
S-adenosylmethionine synthase	METK_LACLA	43 kDa	170	49	3,44
Glycosyltransferase	Q9CJ00_LACLA	38 kDa	15	4	3,44
Uncharacterized protein	Q9CDS4_LACLA	9 kDa	15	4	3,44
Asparagine synthetase	Q9CDJ2_LACLA	72 kDa	32	9	3,42
Probable serine/threonine-protein kinase	PKNB_LACLA	68 kDa	8	2	3,40
Adenylosuccinate synthetase	PURA_LACLA	47 kDa	143	43	3,30
NifU protein	Q9CEP7_LACLA	13 kDa	11	3	3,29
ATP-dependent protease ATP-binding subunit	Q9CHS9_LACLA	90 kDa	66	20	3,24
Nicotinate phosphoribosyltransferase	Q9CGJ6_LACLA	56 kDa	82	25	3,24
Segregation and condensation protein B	SCPB_LACLA	21 kDa	27	8	3,24
50S ribosomal protein L5	RL5_LACLA	20 kDa	14	4	3,22
Uncharacterized protein	Q9CDS3_LACLA	37 kDa	36	11	3,17
Uncharacterized protein	Q9CJ16_LACLA	58 kDa	155	50	3,08
Flotillin-like protein	Q9CHJ2_LACLA	54 kDa	41	13	3,07
Cell division protein ftsA	Q9CEH1_LACLA	49 kDa	91	30	3,00
Uncharacterized protein	Q9CF27_LACLA	38 kDa	10	3	3,00
Transcriptional regulator	Q9CE68_LACLA	15 kDa	10	3	3,00
Tagatose-6-phosphate kinase	Q9CGY4_LACLA	34 kDa	7	2	3,00
Alpha-subunit L-serine dehydratase	Q9CHA7_LACLA	30 kDa	7	2	3,00
Uncharacterized protein	Q9CF30_LACLA	43 kDa	7	2	3,00
Probable nicotinate-nucleotide adenylyltransferase	Q9CIY1_LACLA	23 kDa	7	2	3,00
Uncharacterized protein	Q9CF63_LACLA	64 kDa	7	2	3,00
Lipoprotein	Q9CIN8_LACLA	31 kDa	7	2	3,00
Uncharacterized protein	Q9CEF2_LACLA	18 kDa	243	81	2,99
tRNA uridine 5-carboxymethylaminomethyl modification enzyme	MNMG_LACLA	69 kDa	27	9	2,89
ATP-dependent Clp protease ATP-binding subunit	CLPE_LACLA	83 kDa	481	168	2,86
ATP synthase subunit delta	ATPD_LACLA	19 kDa	18	6	2,85
Endonuclease MutS2	MUTS2_LACLA	87 kDa	15	5	2,82
30S ribosomal protein S4	RS4_LACLA	23 kDa	15	5	2,82

ATP-dependent Clp protease ATP-binding subunit ClpX	CLPX_LACLA	46 kDa	270	96	2,80
UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	MURG_LACLA	39 kDa	9	3	2,71
Uncharacterized protein	Q9CEP4_LACLA	45 kDa	105	39	2,67
Replicative DNA helicase	Q9CHI5_LACLA	50 kDa	53	20	2,61
Uncharacterized protein	Q9CGB4_LACLA	48 kDa	19	7	2,60
Aminopeptidase C	PEPC_LACLA	50 kDa	6	2	2,60
Amino acid aminohydrolase	Q9CGY6_LACLA	42 kDa	6	2	2,60
Mannose-specific PTS system component IID	Q9CEX6_LACLA	34 kDa	6	2	2,60
60 kDa chaperonin	CH60_LACLA	57 kDa	219	84	2,60
Prophage pi2 protein 02	Q9CGT3_LACLA	21 kDa	24	9	2,58
Uncharacterized protein	Q9CG71_LACLA	20 kDa	29	11	2,57
Biotin carboxylase	Q9CHF3_LACLA	49 kDa	52	20	2,56
DNA gyrase subunit A	Q9CGI5_LACLA	93 kDa	11	4	2,56
Asparagine synthetase B	Q9CIK5_LACLA	60 kDa	205	81	2,52
UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1	MURA1_LACLA	46 kDa	94	37	2,52
UvrABC system protein B	UVRB_LACLA	79 kDa	76	30	2,51
Protein RecA, chromosomal	RECA_LACLA	41 kDa	136	54	2,50
30S ribosomal protein S10	RS10_LACLA	12 kDa	50	20	2,46
DNA mismatch repair protein MutS	MUTS_LACLA	94 kDa	13	5	2,45
Diadenosine 5',5'"-P1,P4-tetraphosphate hydrolase	Q9CI07_LACLA	18 kDa	13	5	2,45
Alpha-acetolactate synthase	Q7DAV2_LACLA	61 kDa	164	67	2,44
Riboflavin biosynthesis protein RibBA	Q9CGU7_LACLA	44 kDa	8	3	2,43
Glutamate--tRNA ligase	SYE_LACLA	55 kDa	8	3	2,43
Oligopeptide ABC transporter substrate binding protein	Q9CIL2_LACLA	60 kDa	117	48	2,42
Maltose ABC transporter substrate binding protein	Q9CEZ8_LACLA	44 kDa	292	123	2,37
GTPase HflX	Q9CIY3_LACLA	43 kDa	29	12	2,36
Uncharacterized protein	Q9CDF6_LACLA	22 kDa	10	4	2,33

30S ribosomal protein S7	RS7_LACLA	18 kDa	10	4	2,33
Nitrogen regulatory protein P-II	Q9CF90_LACLA	13 kDa	10	4	2,33
GTPase Der	DER_LACLA	49 kDa	63	27	2,31
Glycine betaine transport ATP-binding protein OpuAA	OPUAA_LACLA	46 kDa	48	21	2,26
Alkyl hydroperoxide reductase	Q9CIL9_LACLA	21 kDa	211	94	2,24
DNA polymerase III subunit beta	DPO3B_LACLA	42 kDa	14	6	2,23
Universal stress protein	Q9CH60_LACLA	16 kDa	14	6	2,23
Formate acetyltransferase	PFL_LACLA	89 kDa	36	16	2,21
ABC transporter ATP binding protein	Q9CGN4_LACLA	72 kDa	18	8	2,18
Spermidine/putrescine import ATP-binding protein PotA	POTA_LACLA	48 kDa	22	10	2,14
Cysteine--tRNA ligase	SYC_LACLA	51 kDa	7	3	2,14
Fumarate reductase flavoprotein subunit	Q9CGH2_LACLA	53 kDa	26	12	2,12
Uncharacterized protein	Q9CED6_LACLA	41 kDa	81	38	2,12
Glucose kinase	Q9CE25_LACLA	34 kDa	9	4	2,11
Ribonuclease Y	RNY_LACLA	60 kDa	11	5	2,09
Acetate-SH-citrate lyase ligase	Q9CGB0_LACLA	39 kDa	11	5	2,09
50S ribosomal protein L14	RL14_LACLA	13 kDa	11	5	2,09
Amino acid ABC transporter substrate binding protein	Q9CDZ9_LACLA	31 kDa	13	6	2,08
Foldase protein PrsA	PRSA_LACLA	34 kDa	13	6	2,08
NH(3)-dependent NAD(+) synthetase	NADE_LACLA	30 kDa	44	21	2,07
S-adenosylmethionine:tRNA ribosyltransferase-isomerase	QUEA_LACLA	39 kDa	75	36	2,07
Thymidylate synthase	TYSY_LACLA	33 kDa	15	7	2,07
Uncharacterized protein	Q9CEI5_LACLA	40 kDa	15	7	2,07
Uncharacterized protein	Q9CIK0_LACLA	15 kDa	15	7	2,07
30S ribosomal protein S5	RS5_LACLA	18 kDa	157	76	2,06
UDP-N-acetylmuramoylalanine--D-glutamate ligase	MURD_LACLA	49 kDa	19	9	2,05
Mannose-specific PTS system component IIAB	Q9CEX8_LACLA	35 kDa	79	39	2,01
Triosephosphate isomerase	TPIS_LACLA	27 kDa	7	15	0,48
Malonyl CoA-acyl carrier protein transacylase	Q9CHF8_LACLA	34 kDa	4	9	0,47

Protein-tyrosine phosphatase	Q9CEK1_LACLA	28 kDa	4	9	0,47
1,4-dihydroxy-2-naphthoyl-CoA synthase	Q9CHK2_LACLA	31 kDa	18	39	0,47
Isoleucine--tRNA ligase	SYI_LACLA	107 kDa	3	7	0,47
3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	FABZ2_LACLA	16 kDa	3	7	0,47
Transcription termination/antitermination protein NusA	Q9CHG7_LACLA	43 kDa	100	220	0,46
Exodeoxyribonuclease A	Q9CHE1_LACLA	38 kDa	6	14	0,45
Dihydrolipoamide acetyltransferase component of PDH complex	Q9CJD7_LACLA	56 kDa	42	95	0,45
Uncharacterized protein	Q9CIG7_LACLA	31 kDa	5	12	0,44
DNA replication protein DnaD	Q9CGM6_LACLA	26 kDa	8	19	0,44
Ribonuclease 3	RNC_LACLA	26 kDa	10	25	0,41
Uncharacterized protein	Q9CE54_LACLA	32 kDa	7	18	0,41
Ferrous ion transport protein A	Q9CJ18_LACLA	17 kDa	8	21	0,40
Uncharacterized protein	Q9CHB7_LACLA	26 kDa	4	11	0,39
Mevalonate kinase	Q9CIF6_LACLA	36 kDa	2	6	0,38
Uncharacterized protein	Q9CEM6_LACLA	31 kDa	2	6	0,38
tRNA dimethylallyltransferase	MIAA_LACLA	34 kDa	2	6	0,38
DNA-directed RNA polymerase subunit omega	RPOZ_LACLA	13 kDa	2	6	0,38
Uncharacterized protein	Q9CIJ7_LACLA	23 kDa	19	52	0,37
Putative gluconeogenesis factor	GNGF_LACLA	36 kDa	3	9	0,37
Uncharacterized protein	Q9CJ34_LACLA	41 kDa	4	12	0,36
Bifunctional protein GlmU	GLMU_LACLA	49 kDa	8	27	0,31
Uncharacterized protein	Q9CHZ8_LACLA	28 kDa	2	9	0,26
Valine--tRNA ligase	SYV_LACLA	101 kDa	3	13	0,26
Leucine--tRNA ligase	SYL_LACLA	94 kDa	3	13	0,26
DNA polymerase III PolC-type	DPO3_LACLA	185 kDa	11	69	0,17
Non-heme chloride peroxidase	Q9CHB2_LACLA	31 kDa		6	0,08
Endoribonuclease YbeY	YBEY_LACLA	19 kDa		7	0,07
Transcriptional regulator	Q9CIF1_LACLA	28 kDa		8	0,06
Uncharacterized protein	Q9CHI3_LACLA	30 kDa		8	0,06
Prophage pi3 protein 57, cI-like repressor	Q9CFN7_LACLA	20 kDa		10	0,05
Pyruvate carboxylase	Q9CHQ7_LACLA	126 kDa	51	2531	0,02

Biotin carboxyl carrier protein of acetyl-CoA carboxylase	Q9CHF5_LACLA	16 kDa		32	0,02

The dashed line indicates the cut off established for specific c-di-AMP interaction assay.

6.2. Bacterial strains

Table XV Bacterial strains

Bacterial Strain	Genotype/Characteristics	Reference
***E. coli* strains**		
E coli 2003	Δ*kdpABC5 kupD1* Δ*trkA*	Hamann 1991 (174)
E coli BL21	*E. coli* B F^{-} *ompT gal dcm lon hsdS$_B$*($r_B^- m_B^-$) [*malB*$^+$]$_{K\text{-}12}$(λ^S)	Studier and Moffatt 1986 (175)
E. coli 0300	*E. coli* 2003 + pIQ310 and pBP370 - Cm^{30} – Amp^{100}	This work
E. coli 0303	*E. coli* 2003 + pIQ310 and pBP373 - Cm^{30} – Amp^{100}	This work
E. coli 0380	*E. coli* 2003 + pWH844 and pBP370 - Cm^{30} – Amp^{100}	This work
E. coli 0383	*E. coli* 2003 + pWH844 and pBP373 - Cm^{30} – Amp^{100}	This work
E. coli 0390	*E. coli* 2003 + pIQ309 and pBP370 - Cm^{30} – Amp^{100}	This work
E. coli 0393	*E. coli* 2003 + pIQ309 and pBP373 - Cm^{30} – Amp^{100}	This work
E. coli DH5α	Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	Hanahan, 1983 (176)
E. coli EC101	*E. coli* JM101 derived. *repA*$^+$ (from pWV01 integrated in chromosome) Km^{40}	Law et al. 1995 (177)
E. coli GP01	*E. coli* BL21 + pIQ402	This work
E. coli GP02	*E. coli* BL21 + pIQ404	This work
E. coli LB08	*E. coli* LB650 + pWH844 - Km^{50} – Cm^{30} – Amp^{100}	This work
E. coli LB09	*E. coli* LB650 + pIQ309 - Km^{50} – Cm^{30} – Amp^{100}	This work
E. coli LB10	*E. coli* LB650 + pIQ310 - Km^{50} – Cm^{30} – Amp^{100}	This work
E. coli LB11	*E. coli* LB650 + pBP372 - Km^{50} – Cm^{30} – Amp^{100}	This work
E. coli LB12	*E. coli* LB650 + pBP371 - Km^{50} – Cm^{30} – Amp^{100}	This work
E. coli LB13	*E. coli* LB650 + pIQ311 - Km^{50} – Cm^{30} – Amp^{100}	This work
E. coli LB650	Δ*kdpABC5* Δ*trkH* Δ*trkG* – Km^{50} – Cm^{30}	Schlösser et al. 1995
E. coli YB01	*E. coli* BL21 + pIQ401	This work
E. coli YB02	*E. coli* BL21 + pIQ403	This work
***E. faecalis* strains**		

E. faecalis JH2-2	Cit^{+}; Rif^{R}; Fus^{R}; plasmid free	Jacob and Hobbs, 1974
***L. lactis* strains**		
L. lactis htrA-clpP	NZ9000 transconjugant carrying *clpP* and *htrA* disruption, plasmid free - Em^{5}	Cortes-Perez 2006
L. lactis IL1403	Derived from IL594 strain. Plasmid free, Trp^{+}	Chopin et al., 1984
L. lactis LL00	*L. lactis* IL1403 + pBV153	This work
L. lactis LL01	*L. lactis* NZ9000 *clpP-htrA* + pNZ8048	This work
L. lactis LL02	*L. lactis* NZ9000 *clpP-htrA* + pNZTS	This work
L. lactis LL03	*L. lactis* IL1403 + pIQ101	This work
L. lactis LL04	*L. lactis* IL1403 + pIQ102	This work
L. lactis LL05	*L. lactis* IL1403 + pIQ103	This work
L. lactis LL06	*L. lactis* IL1403 + pIQ104	This work
L. lactis LL07	*L. lactis* IL1403 *ΔgdpP1s*	This work
L. lactis LL08	*L. lactis* IL1403 *ΔgdpP1b*	This work
L. lactis LL09	*L. lactis* NZ9000 *clpP-htrA* + pIQ101	This work
L. lactis LL10	*L. lactis* NZ9000 *clpP-htrA* + pIQ10TS	This work
L. lactis NZ9000	*L. lactis* MG1363 containing *nisKR* genes integrated into pepN locus, plasmid free	Kuipers 1998
L. lactis NZTS	*L. lactis* NZ9000 + pNZTS	This work

6.3. Plasmids

Table XVI Plasmids

Plasmid	**Description**	**Reference**
pBAD33	arabinose inducible - Cm^{R}	Guzman et al. 1995 (178)
pBP371	pWH844 *ktrC/D L. monocytogenes*	Stülke Lab
pBP372	pWH844 *ktrA/B B. subtilis*	Stülke Lab
pBV153	PcitM promoter, pH inducible Amp^{R} in *E. coli*, Cm^{R} in LAB	Marelli and Magni 2009
pBVGh	Thermosensitive vector for gene deletion Em^{R}	Blancato and Magni 2010
pIQ101	pBV153 + *L. lactis cdaA^{ll}* (Pcit promoter)	This work
pIQ102	pBV153 + *L. lactis cdaA^{ef}* (Pcit promoter)	This work
pIQ103	pBV153 + *L. lactis gdpP^{ll}* (Pcit promoter)	This work
pIQ104	pBV153 + *L. lactis gdpP^{ef}* (Pcit promoter)	This work

pIQ10TS	pIQ101 derived plasmid encoding *cdaA* gene under PcitM promoter and trans-sialidase fragment under Pnis promoter	This work
pIQ309	pWH844 no His-tag + *kupA* IL1403	This work
pIQ310	pWH844 no His-tag + *kupB* IL1403	This work
pIQ311	pWH844 + *kup* JH2-2	This work
pIQ370	pBAD33 + $cdaA^{lmo}$	This work
pIQ373	pBAD33 + *cdaA* D171N Lmo	This work
pIQ401	pWH844 + *yheB L. lactis* NZ9000	This work
pIQ402	pWH844 + *gdpP L. lactis* NZ9000	This work
pIQ403	pWH844 + *yheB L. lactis* IL1403	This work
pIQ404	pWH844 + *gdpP L. lactis* IL1403	This work
pNZ8048	Nisin inducible expression, Cm^R	De Ruyter et al. 1996 (179)
pNZTS	pNZ8048 + codon optimized His_{6x}-tagged trans-sialidase fragment	This work
pUC57-TS	pUC57 + codon optimized His_{6x}-tagged trans-sialidase fragment	This work
pWH844	IPTG inducible – N terminal His tag, Amp^R	Stülke Lab
pWHdnaH	pWH844 no His-tag + RBS + *dnaH*	Grubek, Z. 2016 (180)
pWHezrA	pWH844 no His-tag + RBS + *ezrA*	Grubek, Z. 2016
pWHftsE	pWH844 no His-tag + RBS + *ftsE*	Grubek, Z. 2016
pWHglnP	pWH844 no His-tag + RBS + *glnP*	Grubek, Z. 2016
pWHkupA	pWH844 no His-tag + RBS + *kupA*	Grubek, Z. 2016
pWHkupB	pWH844 no His-tag + RBS + *kupB*	Grubek, Z. 2016
pWHlysP	pWH844 no His-tag + RBS + *lysP*	Grubek, Z. 2016
pWHlysQ	pWH844 no His-tag + RBS + *lysQ*	Grubek, Z. 2016
pWHmvaA	pWH844 no His-tag + RBS + *mvaA*	Grubek, Z. 2016
pWHnah	pWH844 no His-tag + RBS + *nah*	Grubek, Z. 2016
pWHoppA	pWH844 no His-tag + RBS + *oppA*	Grubek, Z. 2016
pWHpdp	pWH844 no His-tag + RBS + *pdp*	Grubek, Z. 2016
pWHprfB	pWH844 no His-tag + RBS + *prfB*	Grubek, Z. 2016
pWHracD	pWH844 no His-tag + RBS + *racD*	Grubek, Z. 2016
pWHrecG	pWH844 no His-tag + RBS + *recG*	Grubek, Z. 2016
pWHrecJ	pWH844 no His-tag + RBS + *recJ*	Grubek, Z. 2016

pWHrecN	pWH844 no His-tag + RBS + *recN*	Grubek, Z. 2016
pWHrelA	pWH844 no His-tag + RBS + *relA*	Grubek, Z. 2016
pWHribH	pWH844 no His-tag + RBS + *ribH*	Grubek, Z. 2016
pWHrpsF	pWH844 no His-tag + RBS + *rpsF*	Grubek, Z. 2016
pWHyciH	pWH844 no His-tag + RBS + *yciH*	Grubek, Z. 2016
pWHyeaD	pWH844 no His-tag + RBS + *yeaD*	Grubek, Z. 2016
pWHyhfC	pWH844 no His-tag + RBS + *yhfC*	Grubek, Z. 2016
pWHyjdJ	pWH844 no His-tag + RBS + *yjdJ*	Grubek, Z. 2016
pWHyncB	pWH844 no His-tag + RBS + *yncB*	Grubek, Z. 2016
pWHyqaB	pWH844 no His-tag + RBS + *yqaB*	Grubek, Z. 2016
pWHyqaD	pWH844 no His-tag + RBS + *yqaD*	Grubek, Z. 2016
pWHyrbD	pWH844 no His-tag + RBS + *yrbD*	Grubek, Z. 2016
pWHyrgI	pWH844 no His-tag + RBS + *yrgI*	Grubek, Z. 2016
pWHyrjB	pWH844 no His-tag + RBS + *yrjB*	Grubek, Z. 2016
pWHytcE	pWH844 no His-tag + RBS + *ytcE*	Grubek, Z. 2016
pWHytjA	pWH844 no His-tag + RBS + *ytjA*	Grubek, Z. 2016
pWHyueF	pWH844 no His-tag + RBS + *yueF*	Grubek, Z. 2016
pWHywfH	pWH844 no His-tag + RBS + *ywfH*	Grubek, Z. 2016

6.4. Oligonucleotides

Homologous sequences hybridizing with template DNA are in italics, restriction sites are underlined. Fwd: forward. Rv: reverse.

Table XVII Oligonucleotides

Name	**Sequence (5’ -> 3’)**	**Purpose**
IQ365	ACAAGTACATATG*ATGCTTGTTGTGGAAC*	(Fwd) *cdaA* - *E. faecalis* JH2-2
IQ366	GCACTAGTAAGCTT*TCATTTTGCGTTTAACC*	(Rv) *cdaA* - *E. faecalis* JH2-2
IQ367	AACTAGACATATG*ATGCAAAAGAAGAGAATTC*	(Fwd) *gdpP* - *E. faecalis* JH2-2
IQ368	CGACTAGTAAGCTT*TCACTCCTGTTCATAC*	(Rv) *gdpP* - *E. faecalis* JH2-2
IQ369	ACGTAACCATATG*TTGACCGACTTCAATC*	(Fwd) *cdaA* - *L. lactis* IL1403
IQ370	GCTCTAGAAAGCTT*TTATTTGCCATTTTTC*	(Rv) *cdaA* - *L. lactis* IL1403
JN371	AAACCATGG*AGAACGTGAAGCAAAAGCACT*	(Fwd) Upstream region of *cdaA* - *L. lactis* IL1403
JN372	TTTAAGCTT*CGAACAAGCGTCATTAATTTTGT*	(Rv) Upstream region of *cdaA* - *L. lactis* IL1403

JN373	AAAAAGCTT*TTGCTCACAATGGAGAATTTTTCG*	(Fwd) Downstream region of *cdaA* - *L. lactis* IL1403
JN374	TTTCCATGG*CAGAAAGATGAACCGTCGCA*	(Rv) Downstream region of *cdaA* - *L. lactis* IL1403
JN375	AAACCATGG*CCGTTTGGGCAATTGAAGACA*	(Fwd) Upstream region of *gdpP* - *L. lactis* IL1403
JN376	TTTAAGCTT*ATTAAAACGGATGACCCCAATTG*	(Rv) Upstream region of *gdpP* - *L. lactis* IL1403
JN377	AAAAAGCTT*ATTATGGAGCAAATGGGTGGG*	(Fwd) Downstream region of *gdpP* - *L. lactis* IL1403
JN378	TTTCCATGG*GCTTTTCTTTTTCCTTAGCTTTGG*	(Rv) Downstream region of *gdpP* - *L. lactis* IL1403
JN379	AAACATATG*GGAGTAATATTATGAATTTTGTCC G*	(Fwd) *gdpP* - *L. lactis* IL1403
JN380	TTTACTAGT*AAGAATACAACTTTCATTTTTCGTT*	(Rv) *gdpP* - *L. lactis* IL1403
JN384	TTTTCTAGA*TTATTTGCCATTTTTCTTTCCTCCT*	(Rv) *cdaA* - *L. lactis* IL1403 (pNZ8048)
JN385	*TATTTGGGAATAAAGCAGTTCACTTAAAA*	(Fwd) *cdaA* operon sequencing
JN386	*GAAAGTAACTGTTCCTATACGTGC*	(Fwd) *cdaA* operon sequencing
JN387	*CAAAAGTCCTGAAATCAAACTGCT*	(Rv) *cdaA* operon sequencing
JN388	*AAAAAAGATAAAAAAGCTCTCCCAATAAG*	(Rv) *cdaA* operon sequencing
JN389	*TGGTTGACATTTTTCTTTGATTGTTATC*	(Rv) *gdpP* IL1403 sequencing
JN400	AAA*CCATGGCCGTTTGGG*	(Fwd) ligation 58 amplification with JN401
JN401	TTT*CCATGGGCTTTTCTTTTTCC*	(Rv) ligation 58 amplification with JN400
JN402	AAA*CCATGGAGAACGTGAAG*	(Fwd) ligation 14 amplification with JN403
JN403	TTT*CCATGGCAGAAAGATGAA*	(Rv) ligation 14 amplification with JN402
JN404	AAA*ATGCGAGCGATGACCAA*	(Fwd) *gdpP* internal fragment - *L. lactis* IL1403

JN405	*TTAATGGCTGTTCGACCGCT*	(Rv) *gdpP* internal fragment - *L. lactis* IL1403
JN406	*GGTTCTATGAAATTTAAAGCAGTGATTT*	(Fwd) gene upstream *gdpP* IL1403 Deletion check
JN407	*TTAGGCCTCGCTAATTTTGACTT*	(Rv) gene downstream *gdpP* IL1403 Deletion check
JN408	AAAACTAGT*AGTTGACCCTGAACGTGAA*	(Fwd) Upstream region of *cdaA L. lactis* NZ9000 / deletion with pBVGh
JN409	TTTGAATTC*AAGCGTCATTAATTTTGTTCCT*	(Rv) Upstream region of *cdaA* L. lactis NZ9000 / deletion with pBVGh
JN410	AAAGAATTC*TAGCACATAATGGCGAGTTT*	(Fwd) Downstream region of *cdaA L. lactis* NZ9000 / deletion with pBVGh
JN411	TTTGAGCTC*TAAATGAACTGTTGCACTGC*	(Rv) Downstream region of *cdaA L. lactis* NZ9000 / deletion with pBVGh
JN414	AAACCATGG*ATGAATTTTGTCCGTCGGTT*	(Fwd) *cdaA* - *L. lactis* IL1403 (pNZ8048)
JN416	*CGATAACGCGAGCATAATAAAC*	(Fwd) pNZ8048 checking
JN417	*GCCTTGGTTTTCTAATTTTGGTTC*	(Rv) pNZ8048 checking
JN418	*GTTTAAACGCTTTGGGACGT*	(Fwd) *recJ* check
JN419	*GTATCGACGTTGACTTGCTT*	(Fwd) *glnP* check
JN500	TTTGGATCC*ATGACCTATCGGATGGTTGA*	(Rv) *pdp* / construction in pWH844 / DRaCALA
JN501	AAAAAGCTT*TTATCTGATAATTTCTAGAATTTCT TTTTGA*	(Fwd) *pdp* / construction in pWH844 / DRaCALA
JN502	AAAGGATCC*ATGGAATTATCAGAAATTAGAAAT TTACTT*	(Fwd) *prfB* / construction in pWH844 / DRaCALA
JN503	TTTAAGCTT*CTACAAGTTCCAACGGAGATA*	(Rv) *prfB* / construction in pWH844 / DRaCALA
JN504	TTTGGATCC*ATGGCATATCAAGCATTATATAGAA AAT*	(Rv) *dnaH* / construction in pWH844 / DRaCALA
JN505	AAAGTCGAC*TTAATCATTAATTTCAACTACTTTT TCACC*	(Fwd) *dnaH* / construction in pWH844 / DRaCALA

JN506	TTTGGATCC*ATGAAATTAACAGATTCAGTGCAATTTTT*	(Rv) *recG* / construction in pWH844 / DRaCALA
JN507	AAAAAGCTT*TCAATCAAAGCCCCCGTCA*	(Fwd) *recG* / construction in pWH844 / DRaCALA
JN508	AAAGGATCC*ATGATAAAAGCAAAATATGATTGGAAA*	(Fwd) *recJ* / construction in pWH844 / DRaCALA
JN509	TTTAAGCTT*TTATTTTTCTAATAAATTTTGATAAATTTCTTT*	(Rv) *recJ* / construction in pWH844 / DRaCALA
JN510	AAAGGATCC*ATGTTACAAGAGATTTCAATCAAAAATTT*	(Fwd) *recN* / construction in pWH844 / DRaCALA
JN511	TTTGTCGAC*TTATTTGCTCAATAAACGTTTAGCC*	(Rv) *recN* / construction in pWH844 / DRaCALA
JN512	AAAGGATCC*ATGGCTGATAAATATAATGTTTTCGA*	(Fwd) *yeaD* / construction in pWH844 / DRaCALA
JN513	TTTAAGCTT*TCAGCGATACAAAAGCTCCA*	(Rv) *yeaD* / construction in pWH844 / DRaCALA
JN514	TTTGGATCC*ATGACTAAATACGAAATTCTTTATATTATTCG*	(Rv) *rpsF* / construction in pWH844 / DRaCALA
JN515	AAAGTCGAC*TTAAGCTTCAACTTTAACGATCATATG*	(Fwd) *rpsF* / construction in pWH844 / DRaCALA
JN516	TTTGGATCC*ATGCCAAAGCAACTTAAAATAAAAG*	(Rv) *yciH* / construction in pWH844 / DRaCALA
JN517	AAAGTCGAC*TTATTCATCAGGTGTCATAATCATTG*	(Fwd) *yciH* / construction in pWH844 / DRaCALA
JN518	AAAGGATCC*ATGAGTATTATAAAATTAAGCAACGTTTC*	(Fwd) *ftsE* / construction in pWH844 / DRaCALA
JN519	TTTAAGCTT*CTAATCATCGTAGCCGTAAACT*	(Rv) *ftsE* / construction in pWH844 / DRaCALA
JN520	TTTGGATCC*ATGAATAAAAAATCGAGTGCTCTTT*	(Rv) *ywfH* / construction in pWH844 / DRaCALA
JN521	AAAAAGCTT*CTACTCCATTATAGTAAGTATCCAGC*	(Fwd) *ywfH* / construction in pWH844 / DRaCALA
JN522	AAAGGATCC*ATGAAGAAATTATTTTCGCTCTGG*	(Fwd) *glnP* / construction in pWH844 / DRaCALA
JN523	TTTAAGCTT*TTATTTCATTCTTTTTTCTACGCGA*	(Rv) *glnP* / construction in pWH844 / DRaCALA
JN524	TTTGGATCC*ATGGAAAACTTCTTTACAATCTTGG*	(Rv) *racD* / construction in pWH844 / DRaCALA

JN525	AAAAAGCTT*TTAATAGTCATCTAATAAATCTTTTTTTCCC*	(Fwd) *racD* / construction in pWH844 / DRaCALA
JN526	TTTGGATCC*ATGAGAAAAAAATTTTATCAAATGTCGC*	(Rv) *mvaA* / construction in pWH844 / DRaCALA
JN527	AAAGTCGAC*TTAGTTTCTGAGACTATTTAATAAACGG*	(Fwd) *mvaA* / construction in pWH844 / DRaCALA
JN528	AAAGGATCC*ATGAAAGTTTCAATATTCTCCACTTG*	(Fwd) *yrjB* / construction in pWH844 / DRaCALA
JN529	TTTAAGCTT*CTATGCTTTGATAAATGCTGGGT*	(Rv) *yrjB* / construction in pWH844 / DRaCALA
JN530	TTTGGATCC*ATGCAAGCAAAATTAGTTAATAAAGTAG*	(Rv) *ytjA* / construction in pWH844 / DRaCALA
JN531	AAAAAGCTT*TTAATGTCTTTGCTCAATATTTTGCT*	(Fwd) *ytjA* / construction in pWH844 / DRaCALA
JN532	TTTGGATCC*ATGCTTCATCTCATTAAAGAAAGC*	(Rv) *yueF* / construction in pWH844 / DRaCALA
JN533	AAAAAGCTT*TTACTTTTCAAGGCTGACCTC*	(Fwd) *yueF* / construction in pWH844 / DRaCALA
JN534	AAAGGATCC*ATGCCTAAAGAACCAGATTTAACC*	(Fwd) *relA* / construction in pWH844 / DRaCALA
JN535	TTTGTCGAC*TTAAGCATTTGTCCGTTTTACAGA*	(Rv) *relA* / construction in pWH844 / DRaCALA
JN536	AAAGGATCC*ATGACAATTATTGAAGGAAATTTAAGAAC*	(Fwd) *ribH* / construction in pWH844 / DRaCALA
JN537	TTTAAGCTT*TTAGCCAATCTTTCTCATTAAATCAA*	(Rv) *ribH* / construction in pWH844 / DRaCALA
JN538	AAAGGATCC*ATGACTGAAGTTTATTTTATTCGACAT*	(Fwd) *yrgI* / construction in pWH844 / DRaCALA
JN539	TTTAAGCTT*TTATTGCTCATAATCAATTTCAATGACT*	(Rv) *yrgI* / construction in pWH844 / DRaCALA
JN540	TTTGGATCC*ATGAGTGAATTAGAGATTCGTAGATT*	(Rv) *yqaB* / construction in pWH844 / DRaCALA
JN541	AAAGTCGAC*CTATTGCTCTTCTAAATTAATCCAGTAA*	(Fwd) *yqaB* / construction in pWH844 / DRaCALA
JN542	AAAGGATCC*ATGGATAACCAAAATCAAGAAATAGG*	(Fwd) *yhfC* / construction in pWH844 / DRaCALA
JN543	TTTAAGCTT*TTATGCGTTTGGATAGTGAATTAAAA*	(Rv) *yhfC* / construction in pWH844 / DRaCALA

JN544	TTTGGATCC*ATGTCAAGTACTGTCATTATCCTC*	(Rv) *ezrA* / construction in pWH844 / DRaCALA
JN545	AAAAAGCTT*TTATAAGTAATCAGGAGTAGGTTTATTTTT*	(Fwd) *ezrA* / construction in pWH844 / DRaCALA
JN546	AAAGGATCC*ATGGCATTTATCACCGAAAAAC*	(Fwd) *ytcE* / construction in pWH844 / DRaCALA
JN547	TTTAAGCTT*TTAAATTTCTGATTTTGTAAATACACCG*	(Rv) *ytcE* / construction in pWH844 / DRaCALA
JN548	TTTGGATCC*ATGGCTTTAACGATCGAACG*	(Rv) *yqaD* / construction in pWH844 / DRaCALA
JN549	AAAAAGCTT*TTAAAATCCCAAGTTTATTCCAGGC*	(Fwd) *yqaD* / construction in pWH844 / DRaCALA
JN550	TTTGGATCC*ATGAAATTAAAAGATATAGAGAAATCAACAAA*	(Rv) *yrbD* / construction in pWH844 / DRaCALA
JN551	AAAAAGCTT*TTATTTTTCATTTAAAACCTGACGAAG*	(Fwd) *yrbD* / construction in pWH844 / DRaCALA
JN552	AAAGGATCC*ATGTTTGGAAAGTTTAATCGCTC*	(Fwd) *yjdJ* / construction in pWH844 / DRaCALA
JN553	TTTAAGCTT*TCATTTATTTTTTCTATTATTTCCTTCTAAGG*	(Rv) *yjdJ* / construction in pWH844 / DRaCALA
JN554	TTTGGATCC*ATGAATGATATTTTACAACTCACAATTG*	(Rv) *nah* / construction in pWH844 / DRaCALA
JN555	AAAGTCGAC*TTAATTATCATATTTTTTCAAGACCATTTTAATT*	(Fwd) *nah* / construction in pWH844 / DRaCALA
JN556	AAAGTCGAC*TTGAGTCTGAGTCAAACAAGTC*	(Fwd) *lysP* / construction in pWH844 / DRaCALA
JN557	TTTAAGCTT*TTAATCTTTATGACGACTTAAATCAACC*	(Rv) *lysP* / construction in pWH844 / DRaCALA
JN558	AAAGGATCC*ATGGAAAATCAAAATCAGGTCAAG*	(Fwd) *lysQ* / construction in pWH844 / DRaCALA
JN559	TTTAAGCTT*TTATTTTTCCCGACTCAAATCTACT*	(Rv) *lysQ* / construction in pWH844 / DRaCALA
JN560	AAAGGATCC*ATGGGTTATGAATCTAATCGCTC*	(Fwd) *kupA* / construction in pWH844 / DRaCALA
JN561	TTTGTCGAC*TTATGATTCTCCTTGATTTTCTTCTG*	(Rv) *kupA* / construction in pWH844 / DRaCALA
JN562	AAAGGATCC*GTGGGTCAAGTACACTTACATAA*	(Fwd) *kupB* / construction in pWH844 / DRaCALA

JN563	TTTGTCGAC*TTAATGAGTTGCTGAAGCTTCT*	(Rv) *kupB* / construction in pWH844 / DRaCALA
JN564	AAAGGATCC*ATGAACTTATTAAAAACAAACTGG GA*	(Fwd) *yncB* / construction in pWH844 / DRaCALA
JN565	TTTAAGCTT*TTATTTTTCATAATATTTATCTAAAA TCTCCATTTT*	(Rv) *yncB* / construction in pWH844 / DRaCALA
JN566	TTTGGATCC*ATGAAAAAATTAAAAGTAACTTTAT TGGCA*	(Rv) *oppA* / construction in pWH844 / DRaCALA
JN567	AAAAAGCTT*CTATTTGGTTGCCATCTTATCAGA*	(Fwd) *oppA* / construction in pWH844 / DRaCALA
JN568	AAAGGATCC*ATGATAAAAGCAAAATATGATTGG AAAG*	(Fwd) *recJ* / construction in pWH844 / DRaCALA
JN569	TTTAAGCTT*ACGAAATTATTTTTCTAATAAATTTT GATAAATTTC*	(Rv) *recJ* / construction in pWH844 / DRaCALA
JN581	*AGGTTATATTCATGACCTTTGGAAA*	(Fwd) *relA* check
JN586	*CGCTTGTTCGTGGTGTGATT*	(Fwd) internal fragment *cdaA* - *L. lactis* IL1403
JN587	*CGTAAGCTGAAATATGCCCATCA*	*(Rv)* internal fragment *cdaA* - *L. lactis* IL1403
IQ668	AAAGGATCC*AAGGTACCAATGAACGAAATTTTA GAAAAAATAAAAGC*	(Fwd) *yheB* - *L. lactis* IL1403 (pWH844)
IQ669	TTTGGATCCC*TGCAGTTATTTTAAATTATCTTGT AATTCTTGCC*	(Rv) *yheB* - *L. lactis* IL1403 (pWH844)
IQ670	TTTGGATCC*TTGGTACCAATGAACGAAATTTTAG AAAAAATTAAAGCC*	(Rv) *yheB* - *L. lactis* NZ9000 (pWH844)
IQ671	AAAGGATCC*CTGCAGTTATTTCAAGTTATCTTGT AACTCTTG*	(Fwd) *yheB* - *L. lactis* NZ9000 (pWH844)
IQ682	AAAGAATTC*ATTAAAGAGGAGAAAATTAGAATG GGTTATGAATCTAATCG*	(Fwd) RBS+*kupA L. lactis* IL1403 (pWH844)
IQ683	AAAGAATTC*ATTAAAGAGGAGAAAACAAAAGTG GGTCAAGTACAC*	(Fwd) RBS+*kupB L. lactis* IL1403 (pWH844)
IQ684	AAAAGATCT*AGGTTTTTATATTACAGCTCCAGG A*	(Fwd) PcitM+*cdaA* from pIQ101
IQ685	TTTAGATCT*CTGGTATCTTTATAGTCCTGTCG*	(Rv) PcitM+*cdaA* from pIQ101
IQ687	AAAGGATCC*GTGTTACACAAAGCAGAGGGG*	(Fwd) *kup* - *E. faecalis* JH2-2

IQ688	TTTGTCGAC*ATTTCTTCTATTTATGAACGATTCTTTC*	(Rv) *kup* - *E. faecalis* JH2-2
IQ696	AAACTGCAG*GTTGAAGAAGGTTTTTATATTACAGC*	(Fwd) hisTScf
IQ697	TTTGTCGAC*GGTGGACAAATTTACATTAGTCTC*	(Rv) hisTScf
IQ706	*ACAGGTACTGGCGCTAAAAAC*	(Fwd) External check (*E. faecalis* JH2-2 *gdpP* deletion)
IQ707	*GCTACGACATGAACCTTAATTTTC*	(Rv) External check (*E. faecalis* JH2-2 *gdpP* deletion)
IQ708	*CGGCTCAAACAAACCTAGATAC*	(Fwd) Internal check (*E. faecalis* JH2-2 *gdpP* deletion)
IQ709	*CTTTTGACATCGGGAATGATTTC*	(Rv) Internal check (*E. faecalis* JH2-2 *gdpP* deletion)

6.5. Materials

6.5.1. Buffers used in this work

Table XVIII Buffers routinely used

Solution	Composition	Concentration
TAE	Tris-HCl pH 8.0 EDTA Acetic acid	40 mM 10 mM 0.057 %
TE	Tris-HCl pH 8.0 EDTA	10 mM 1 mM
Wash (W)	Tris-HCl pH 7.5 NaCl	100 mM 150 mM
Lysis buffer	Tris-HCl Urea Adjust to pH 8.0	30 mM 8 M
Sample Buffer	Tris-HCl pH 7.0 Dithiothreitol (DTT) β-mercaptoethanol SDS Bromophenol blue Glycerol	50 mM 0.5 mM 4 % (v/v) 2 % (p/v) 0,005 % (p/v) 15 % (v/v)
Running buffer	Tris Base pH 8.3 Glycine (pH 8,3) EDTA	25 mM 192 mM 1 mM

	SDS	0,1 % (p/v).
Buffer C	Tris–HCl pH 7.4 NaCl Glycerol	50 mM 500 mM 5 %
PBS	NaCl KCl Na_2HPO_4 KH_2PO_4 Adjust pH to 7.3	8 g/l 0.2 g/l 1.44 g/l 0.24 g/l
CP1 buffer	$MgCl_2$ Tris-HCl pH 8.0 NaCl DTT Protease inhibitor tablet	1 mM 5 mM 230 mM 0.5 mM 1 unit
CP2 buffer	CP1 buffer glycerol BSA EDTA	1X 10 % 50 µg/ml 0.004 %
Buffer E	Tris-HCl pH 8.0 NaCl EDTA D-desthiobiotin	100 mM 1 mM 1 mM 5 mM

6.5.2. Chemicals

Acetic acid	ChemSolute/Merck
Acryl:Bisacrylamide	Roth
L-Arabinose	Sigma-Aldrich
c-di-AMP	Biolog
Agar	Roth
Agarose	PEQLAB/Genbiotech
$AgNO_3$	Roth
Ammonium peroxydisulfate	Roth
Ampicillin	Roth
BCIP	Sigma-Aldrich
Biotinylated c-di-AMP	Biolog
Bromophenol blue	Riedel-de Haën
BSA	AppliChem
$CaCl_2$ $2H_2O$	Roth

Casamino acids (CAA)	Roth
CDP-Star	Roche
Chloramphenicol	Serva/Sigma-Aldrich
Coomassie Brilliant Blue, G-250	Roth
DAPI	AppliChem
D-desthiobiotin	IBA
DMSO	Roth
dNTPs	Thermo Scientific
Ethanol	VWR Prolabo chemicals/Merck
Formaldehyde	Roth
D-(+)-Glucose	AppliChem
L-glutamic acid	Roth
Glycerol	Roth/Merck
Glycine	AppliChem/Sigma-Aldrich
c-di-NMP	Sigma-Aldrich
HCl	Biolog
HDGreen Plus DNA stain	Intas
Imidazole	Sigma-Aldrich
Isopropyl β-D-1-thiogalactopyranoside	PEQLAB/Sigma-Aldrich
Kanamycin	AppliChem
KCl	Roth/Merck
KH_2PO_4	Roth/Merck
$K_2HPO_4 \cdot 3H_2O$	Roth/Merk
KOH	Roth/Merck
λ-DNA	Thermo Scientific
β-mercaptoethanol	Roth
Methanol	VWR Chemicals/Merck
$MgCl_2 \cdot 3H_2O$	Roth/Sigma-Aldrich
$MgSO_4 \cdot 7H_2O$	Roth/ Sigma-Aldrich
$MnCl_2 \cdot 4H_2O$	Roth/ Sigma-Aldrich
NaCl	Roth/ Sigma-Aldrich
Na_2CO_3	Roth/ Sigma-Aldrich
Na_2-EDTA · 2H2O	Roth/ Sigma-Aldrich
$(NH_4)_2SO_4$	Fluka
NaOH	Roth/ Sigma-Aldrich
$Na_2S_2O_3 \cdot 5H_2O$	Merck

Ni^{2+}-NTA Sepharose	Sigma-Aldrich
NBT	IBA ICN
PageRulerTM Plus Protein Ladder	Thermo Scientific
Protease Inhibitor Tablet	Sigma
Sodium dodecyl sulfate (SDS)	Roth
Strep-Tactin Sepharose	IBA
D-(+)-Sucrose	AppliChem/Sigma-Aldrich
Tris	Roth/Sigma-Aldrich
Tryptone	Roth/Oxoid
L-Tryptophan	AppliChem
Tween-20	Sigma-Aldrich
Yeast extract	Oxoid
X-Gal	Sigma-Aldrich
Xylene cyanol	Merck

6.5.3. Auxiliary material

Centrifuge cups	Beckmann
Cuvettes (microlitre, plastic)	Greiner
Eppendorf tubes	Greiner
Falcon tubes	Starstedt/
Glass beads 0.1 mm	Roth
Glass pipettes	Brad
Micropipettes	Eppendorf
Petri dishes	Greiner
Pipette tips	Greiner
Poly-prep chromatography columns	BioRad
PVDF membrane	BioRad
Nitrocellulose membrane	GE Healthcare
Vivaspin® turbo 15	Sartorius

6.5.4. Instrumentation

ÄKTAprime plus	GE Healthcare
Autoclave LTA 2x3x4	Zirbus Technology

AxioCam MRm	Zeiss
AxioImager M2 Microscope	Zeiss
Axioskop 40 Microscope	Zeiss
Gene Pulser Xcell and PC Module	Bio-Rad
Centrifuge Heraeus FRESCO 21	Thermo Scientific
Centrifuge Heraeus Megafuge 16R	Thermo Scientific
Centrifuge Sorvall RC 6+	Thermo Scientific
Centrifuge Sorvall WX Ultraseries	Thermo Scientific
Chrom. Data System LC-NetII/ADC	Jasco
ECPlan-NEOFLUAR 100X/1.3	Zeiss
French pressure cell press	Thermo Scientific
Horizontal shaker 3006	GFL
Microplate reader	BioTek
Ice machine MF 36	Scotsman
Incubator Heraeus	Thermo Scientific
Incubator shaker	Innova®44 series Eppendorf
Labcycler	SensoQuest
Magnetic stirrer KMO 2 basic	IKA-Werke
Mini PROTEAN® System	BioRad
Molecular Imager® Gel Doc™XR+	BioRad
NanoDrop ND-1000	PeqLab Biotechnologie
pH-meter Calimatic 766	Knick
Plan Neofluar 100x/1.30	Zeiss
Plan Neofluar 40x/0.75	Zeiss
Power Pac Basic™	BioRad
Press machine G.	Heinemann
Scale CP22025	Sartorius
Ultrospec 2100 pro spectroph.	Amersham Biosciences
UV Detector UV-2075Plus	Jasco
Sterile bench HERA safe KS12	Thermo Scientific
HiLoad 16/600 Superdex 200 pg	GE Healthcare
ThermoStat Plus	Eppendorf
Tyssue Lyser II	QIAGEN
Vortex Schütt	Labortechnik

Water desalination plant	Millipore

6.5.5. Commercial systems

DNeasy® Blood & Tissue Kit	QIAGEN
NucleoSpin® Plasmid	Macherey-Nagel
peqGOLD Bacterial DNA kit	PEQLAB
QIAquick® PCR purification Kit	QIAGEN
Roti®-Quant	Roth

6.5.6. Enzymes and antibodies

Alkaline phosphatase (AP)	Thermo Scientific
DNase I	Roche
Phusion DNA polymerase	Thermo Scientific
RNase A	Roche
Restriction enzymes	Thermo Scientific
T4-DNA ligase	Thermo Scientific
Anti-Rabbit IgG	Promega

6.6. Informatic tools

6.6.1. Websites

Tabla XIX Websites

URL	Provider	Purpose
http://biotools.nubic.northwestern.edu/OligoCalc.html	Northwestern University, USA	Pimer properties analysis
https://www.ncbi.nlm.nih.gov/pubmed/	National Institute of Health, Bethesda, USA	Literature
http://www.uniprot.org/	European Bioinformatic Institute (EMBL-EBI), Swiss Institute of Bioinformatic (SIB), Protein Information Resource (PIR)	Protein information and analysis

http://www.insilico.uni-duesseldorf.de/Lig_Input.html	Düsseldorf University	Insert:Vector ratio calculation in PCR
https://www.expasy.org/	Swiss Institute of Bioinformatics (SIB)	Protein topology prediction
http://wlab.ethz.ch/protter/start/	Wollscheid Laboratory	Protein topology prediction
http://subtiwiki.uni-goettingen.de/	General Microbiology department, Göttingen University	Literature about *B. subtilis*

6.6.2. Softwares

Table XX Softwares

Software	**Provider**	**Purpose**
Microsoft Office 2010	Microsoft Inc.	Primer properties analysis
ImageLab™ Software	BioRad	Image processing
EndNote	Clarivate analytics	References
SigmaPlot	Systat software, Inc	Plotting
ChemoStar Imager	Intas	Image processing
Image J 1.49p	NIH	Image processing
AxioVision Rel. 4.8	Zeiss	Image Uptake
Geneious 7.0.2	Biomatters Ltd	Bioinformatics

6.7. Abbreviations

Tabla XXI Abbreviations

General abbreviations	
% (vol/vol)	% (volume/volume)
Amp^r	Ampicillin Resistance
APS	Ammonium persulfate
ATP	adenosine triphosphate
Au	Absorption units
Biovar.	Biovariety
BSA	Bovine Serum Albumin

CAA	Casaminoacids
CE	Crude Extract
Cm^r	Chloramphenicol resistance
dH_2O	Deionized water
DNA	Deoxyribonucleic acid
DNase	DNA nuclease
dNTP	desoxyribonucleoside- triphosphate
DTT	Dithiothreitol
EDTA	N, N, N', N'- ethylenediaminetetraacetic acid
Em^r	Erithromycin resistance
FDA	Food and Drug Administration
Fig.	figure
FT	Flowthrough
Fwd	forward
IPTG	Isopropyl thiogalactopyranoside
Km^r	Kanamycin Resistance
LAB	Lactic Acid Bacteria
LB	Luria Bertani
Max	maximum
MWM	Molecular Weight Marker
NAD	Nicotinamide Adenine Dinucleotide (oxidized)
NADH	Reduced Nicotinamide Adenine Dinucleotide
Ni^{2+}-NTA	nickel-nitrilotriacetic acid
NICE	Nisin Control Expression System
NIH	National Institute of Health
Nis	Nisin
NTP	ribonucleoside triphosphate
ODx	Optical density, measured at the wavelength λ = x nm
P	promoter
PBS	Phosphate buffer saline
PCR	Polymerase Chain Raction
pH	Power of hydrogen
rbs	Ribosome Binding Site
rpm	Revolutions per minute
Rv	Reverse

SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis with SDS
Sn	Supernatant
Spp	Species
TEMED	N,N,N',N'- tetramethylethyldiamine
Tris	tris(hydroxymethyl)amino- methane
WT	wild type
X-Gal	5-bromo-4-chloro- indolylgalactopyranoside
Units	
A	Ampere
bp	base pairs
Da	Dalton
g	gram
h	hour
Lt.	liter
m	meter
M	molar
min	minute
Mol	mol
V	volt
ºC	degree Celsius
Prefixes	
K	kilo
m	milli
µ	micro
n	nano
Nucleosides	
A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil
Amino acids nomenclature	
A	Ala
C	Cys

D	Asp
E	Glu
F	Phe
G	Gly
H	His
I	Ile
K	Lys
L	Leu
M	Met
N	Asn
P	Pro
Q	Gln
R	Arg
S	Ser
T	Thr
V	Val
W	Trp
Y	Tyr

Curriculum vitae

PERSONAL INFORMATION

Name: Ingrid María Quintana

Date of birth: March 27th 1990

Place of birth: Rosario, Santa Fe (Argentina)

Nationality: Argentina

EDUCATION AND TRAINING

09/14 – 04/18 **PhD in Molecular Biosciences and Biomedicine**

Universidad Nacional de Rosario (UNR – Argentina) – Georg-August-Universität (Göttingen – Germany)

Title of thesis: Cyclic di-nucleotide monophosphate cyclase in Firmicutes: from basic to practical approach. Instituto de Biología Molecular y Celular de Rosario (IBR – CONICET) – Institute for Genetics and Microbiology (Department of General Microbiology (Georg-August-Universität)

04/13 – 08/14 **PhD in Biological Sciences**

Universidad Nacional de Rosario (UNR – Argentina)

Title of thesis: Cyclic di-nucleotide monophosphate cyclase in Firmicutes: from basic to practical approach. Instituto de Biología Molecular y Celular de Rosario (IBR – CONICET)

04/08 – 03/13 **Licenciatura en Biotecnología (B.Sc. in Biotechnology)**

Facultad de Ciencias Bioquímicas y Farmacéuticas – Universidad Nacional de Rosario

PUBLICATIONS

Martino GP, Quintana IM, Espariz M, Blancato VS, Magni C. Aroma compounds generation in citrate metabolism of *Enterococcus faecium*: Genetic characterization of type I citrate gene cluster. Int J Food Microbiol. 2016 Feb 2;218:27-37.

Martino GP, Quintana IM, Espariz M, Blancato VS, Gallina Nizo G, Esteban L, Magni C. Draft Genome Sequences of Four *Enterococcus faecium* Strains Isolated from Argentine Cheese. Genome Announc. 2016 Feb 4;4(1). pii: e01576-15.

Quintana IM, Grubek Z., Gibhardt J., Lee V. T., Hammer E., Commichau F. M., Stülke J., and Magni C. The KupA and KupB proteins of *Lactococcus lactis* IL1403 are c-di-AMP receptor proteins responsible for potassium uptake. (2018) Applied and Environmental Microbiology. Sent.

Quintana IM, Espariz M., Stülke J., Villar S., González F., Pacini F., Cabrera G., Bontempi I., Procheto E., Perez A. R., Marcipar I., Blancato V., and Magni C. Genetic engineering of *Lactococcus lactis* co-producing antigen and the mucosal adjuvant 3´ 5´- cyclic di adenosine monophosphate (c-di-AMP) to design a mucosal vaccine prototype. (2018) Frontiers in Microbiology. Sent.