Analysis of Quorum Sensing Regulatory Systems in the Human Isolate *Lactobacillus plantarum*

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Contents

Abstract

Bacteria can interact with each other via specific cell-to-cell communication systems that are used to regulate the expression of genes involved in diverse functions, such as virulence, genetic competence, or production of antimicrobial compounds. Modulation of the genes involved is done in a co-ordinated and cell density-dependent way and has been termed quorum sensing (QS). Regulation of QS systems is mediated via specific extracellular signaling molecules, which in Gram-positive bacteria generally are autoinducing peptides (AIPs). QS systems are expected to evolve in high cell-density ecosystems with many microbial interactions, where they would enable bacterial populations to co-ordinate responses that improve their competitiveness, adaptation to changing environmental conditions, or interactions between bacteria and their abiotic or biotic environment. *Lactobacillus plantarum* is a lactic acid bacterium (LAB) that is encountered in ecological niches like fermented food products or plant material, but it is also a natural inhabitant of the human gastrointestinal tract (GI-tract). Intestinal LAB face large changes in environmental conditions during GI-tract passage and inhabit densely populated GI-tract compartments, while in the nutrient-rich industrial and food environments LAB can grow to high cell densities. The characteristics of these natural environments are therefore expected to be favorable for the development and presence of QS systems in LAB. This thesis describes the functional analysis of genes that were annotated as QS systems on the completed genome sequence of *L. plantarum*. A combination of functional genomics and bioinformatics approaches was used to predict and confirm their putative QS function. Using a comparative genomics approach 5 two-component regulatory systems (TCS), consisting of a histidine protein kinase (HPK) and response regulator (RR), which could be involved in peptide-based QS were predicted from the genome sequence. Functional analysis of one of these TCS, designated *Lactobacillus agr*-like module (*lam*), indicated its putative regulatory function in adherence and identified an AgrD-like AIP encoded by it. Mutational analysis of a gene coding for a transcriptional regulator that contained an AraC-type DNA-binding domain and was flanked by a HPK-encoding gene, showed its regulatory role in adherence and cell-aggregation. The results demonstrate the potential of genomics and molecular tools for elucidating the functions of uncharacterized QS genes and identifying new AIPs in the *L. plantarum* genome, as well as their implications on future QS research in lactobacilli.

General introduction

Introduction

Since a decade it is known that bacteria can interact with each other via specific communication systems (9). These cell-to-cell communication mechanisms are used to regulate the expression of genes involved in diverse functions, such as bioluminescence, virulence, genetic competence, or the production of antimicrobial compounds (17, 33). Modulation of the genes involved is done in a co-ordinated and cell density-dependent manner and has been termed quorum sensing (9). Regulation of these quorum sensing systems is mediated via specific extracellular signaling molecules, which in Gram-negative bacteria often are *N*-acyl homoserine lactones (33), while peptides are the most common signaling molecules in Gram-positive bacteria (26). Quorum sensing systems are expected to evolve in high cell-density ecosystems with many microbial interactions and a diffusion barrier, where they would enable bacterial populations to co-ordinate responses that might lead to competitive advantages, efficient adaptation and responses to changing environmental conditions, or co-ordination of interactions between bacteria and their abiotic or biotic environment.

The mammalian gastro-intestinal (GI) tract is a complex and dynamic microbial ecosystem, which is divided into well-defined anatomical regions with different environmental conditions with respect to pH, redox potential and transition times of intestinal contents. The microbial community changes concomitant with these conditions with a strong increase in both microbial density and diversity going from stomach to colon (19). Classical and molecular approaches (27, 34) revealed that the GI-tract microbiota consists of over 1000 largely anaerobic species, among which many Gram-positive bacteria, of which only a small fraction has been isolated in pure culture (30). Assembly of the GI-tract microbiota starts with the colonization of the gut at birth, after which the microbial composition develops and a complex climax community is established at very high cell densities (8, 19). This climax community in adults shows a stable and host-specific composition, which appears to be the end result of microbe-host and microbe-microbe interactions (15).

Lactic acid bacteria (LAB) are Gram-positive bacteria that are common inhabitants of the human GI-tract microbiota (31) and also generally used in food fermentations of (for example) dairy, meat and vegetable raw materials (4, 23). Intestinal LAB often face large changes in environmental conditions during GI-tract

passage and inhabit densely populated GI-tract compartments, such as the small and large intestine, while in the nutrient-rich industrial and food environments LAB are able to grow to high cell densities. The characteristics of these natural environments are therefore expected to be favorable for the development and presence of quorum sensing systems in LAB. Typical intestinal LAB include genera within the low G + C *Clostridium* phylum, such as *Lactobacillus*, *Pediococcus* and *Leuconostoc* (31). Among the LAB, in particular ingested strains from the genus *Lactobacillus* are implicated in providing probiotic properties and to promote health in man and animals (22). *Lactobacillus plantarum* is a flexible and versatile species that is encountered in diverse environmental niches like fermented food products or on plant material (5, 7). Moreover, *L. plantarum* is a natural inhabitant of the human GI-tract (1) and some *L. plantarum* strains are marketed as a probiotic (21). *L. plantarum* WCFS1 is a single colony isolate of *L. plantarum* NCIMB8826, which was originally isolated from human saliva (13), and was shown to survive stomach passage and persist in the human GI-tract after a single dosage (32).

General features of the *Lactobacillus plantarum* WCFS1 genome

The *L. plantarum* WCFS1 genome sequence has been completed and its 3.3 Mb genome is among the largest genomes known in LAB (16, 18). By far the largest class of proteins encoded on the genome is represented by transporters (416 genes), among which 195 primary active transporters (including 147 ATP-binding cassette (ABC) transporters) were identified (Table 1.1). Many of these importers transport amino acids and peptides, but the genome also contains a high number of genes encoding putative sugar transporters that include 57 complete as well as several incomplete sugar phosphotransferase systems (PTS). This number of PTS is much higher than that on average found in other microbial genomes, and similar only to *Listeria monocytogenes* (11) and *Enterococcus faecalis* (24). In addition to these PTS, the *L. plantarum* genome encodes 30 transporter systems that were predicted to be involved in transport of carbon sources. This large set of genes involved in sugar uptake and utilization, indicates that *L. plantarum* is adapted to the efficient utilization of many different sugars. Many of the genes for sugar transport and metabolism are clustered near the origin of replication in a region that encodes almost exclusively proteins for sugar transport, metabolism, and regulation (between 2.70 to 2.85 and 3.10

to 3.29 Mb of the WCFS1 chromosome) (20). This entire region has a lower G + C content (41.5%) than the rest of the genome (44.5%), suggesting that these genes have been acquired by horizontal gene-transfer. This part of the *L. plantarum* chromosome therefore was proposed to represent a lifestyle-adaptation region that is used to effectively adapt to changing environmental conditions *L. plantarum* encounters (20). For amino acid uptake and utilization the *L. plantarum* genome encodes peptide uptake systems, but not the extracellular protease Prt required for primary breakdown of proteins. *L. plantarum* has 19 genes encoding intracellular peptidases, but despite this elaborate protein-degradation machinery the genome encodes complete pathways for the biosynthesis of most amino acids. Only the pathways leading to the branched-chain amino acids valine, leucine, and isoleucine are absent. The extensive protein-degradation machinery creates a selective advantage for growth in the protein-rich environments that LAB generally inhabit, such as the GI-tract or dairy products, while the protein-biosynthesis machinery supports growth in environments providing less proteins.

The genome was predicted to encode 217 extracellular proteins with N-terminal signal sequences, of which 144 contain potential signal peptidase cleavage sites. Most of these proteins are predicted to be anchored to the cell by single N- or C-terminal transmembrane anchors (83 proteins), lipoprotein anchors (47 proteins), LPxTG-type anchors (25 proteins), or other cell-wall binding (repeated) domains. Most of the predicted extracellular enzymes are hydrolases, many of which have unknown specificity. In general, the extracellular proteins have a normal signal peptide and multiple domains, including at least one cell-envelope anchoring domain. It is highly likely that some of these extracellular proteins play a role in adhesion or binding to other cells or proteins, because they contain domains with homology to proteins in databases with predicted functions such as mucus-binding, fibronectin-binding, aggregation-promoting, intercellular adhesion, or cell clumping. Interestingly, the genes encoding extracellular proteins are not randomly distributed over the chromosome and many of these genes appear in clusters of three to six genes. The region of the chromosome harboring these clusters, is adjacent to the region that almost exclusively encodes proteins involved in sugar transport and utilization and the regulation thereof (the above-mentioned life-style adaptation islands). These findings support the hypothesis that this part of the *L. plantarum* genome represents a lifestyle-adaptation region that encodes over-represented functions related to interactions with

varying environments, and aids to its persistence and survival in diverse ecological niches (18, 20).

Regulatory genes on the *Lactobacillus plantarum* WCFS1 genome

Another large class of genes is predicted to have regulatory functions, and contains 261 genes (8.5% of total proteins), among which three sigma-factor encoding genes (*rpoD*, *rpoN*, and *sigH*), over 200 transcriptional regulators and 31 two-component regulatory genes (of the latter 13 genetically linked pairs of histidine protein kinases and response regulators are predicted) (Table 1.1). The relatively high proportion of regulatory genes found in *L. plantarum* is only similar to that found in other, versatile, adaptive species like *Pseudomonas aeroguinosa* (8.4%) and *Listeria monocytogenes* (7.3%), and therefore is probably a reflection of the many different environmental conditions that these bacteria face. In comparison, the more related *Lactobacillus johnsonii* and *Lactobacillus acidophilus* genomes both encode only 114 putative regulators (both 6% of total proteins), which reflects the more stable, nutrient-rich, gastrointestinal environment where these organisms reside (2, 3, 25). Many of the transcriptional regulators and two-component regulatory genes (TCS) are signal transduction systems that detect (specific) environmental signals, such as nutrients (C, N, P, metal ions) (10, 12), catabolites (28) or xenobiotic compounds (29), but also monitor and respond to physico-chemical changes in osmolarity, temperature or pH (6, 14). In addition, especially in Gram-positive species, TCS are also used to respond to specific secreted signaling molecules in quorum sensing (26).

Table 1.1 Distribution of predicted ORFs over functional classes (numbers based on the *L. plantarum* WCFS1 genome-update version 5)

Class	Number of ORFs	%
Hypothetical proteins	918	29.95
Transport and binding proteins	416	13.57
Regulatory functions	261	8.52
Energy metabolism	240	7.83
Cell envelope	232	7.57
Other categories	206	6.72
Protein synthesis	145	4.73
Cellular processes	120	3.92
Purines, pyrimidines, nucleosides and nucleotides	89	2.90
DNA metabolism	82	2.68
Amino acid biosynthesis	83	2.71
Biosynthesis of cofactors, prosthetic groups, and carriers	68	2.22
Fatty acid and phospholipid metabolism	61	1.99
Central intermediary metabolism	59	1.92
Protein fate	57	1.86
Transcription	28	0.91
total	**3065**	**100**

Outline of this thesis

The research described in this thesis focuses on the functional analysis of genes that were annotated as quorum sensing regulatory systems on the sequenced genome of the humane isolate *Lactobacillus plantarum* WCFS1. A combination of functional and comparative genomics, as well as bioinformatics approaches was used to predict and confirm their putative functions.

The current chapter **(Chapter 1)** describes general features of GI-tract microbiota and the genome of the human isolate *Lactobacillus plantarum* WCFS1.

In **Chapter 2** a general overview on peptide-based quorum sensing in Gram-positive bacteria is given. Special attention is given to the impact of genomics on quorum sensing studies.

In **Chapter 3** a comparative genomics approach is used to predict two-component regulatory systems (TCS) on the completed *L. plantarum* WCFS1 genome sequence that could be involved in quorum sensing. Two of these putative quorum sensing TCS are further analyzed in Chapters 4 and 5.

Chapter 4 describes the functional analysis of the *lamBDCA* TCS gene-cluster, and discusses results indicating the putative regulatory function of *lamBDCA* as well as the identification of an autoinducing peptide encoded by it.

Chapter 5 describes the analysis of a transcriptional regulator containing an AraC-type DNA-binding domain, and its regulatory role in adherence phenotypes.

Chapter 6 summarizes the general conclusions on the research results described in this thesis and shows future perspectives for the research field discussed.

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Cell to cell communication by autoinducing peptides in Gram-positive bacteria

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Abstract

While intercellular communication systems in Gram-negative bacteria are often based on homoserine lactones as signalling molecules, it has been shown that autoinducing peptides perform this role in cell-to-cell communication in Gram-positive bacteria. Many of these peptides are exported by dedicated systems, posttranslationally modified in various ways, and finally sensed by other cells via membrane-located receptors that are part of two-component regulatory systems. In this way the expression of a variety of functions including virulence, genetic competence, biofilm formation, and the production of antimicrobial compounds can be modulated in a co-ordinated and cell density- and growth phase-dependent manner. Occasionally the autoinducing peptide has a dual function, such as in the case of nisin that is both a signalling pheromone involved in quorum sensing and an antimicrobial peptide. Moreover, biochemical, genetic and genomic studies have shown that bacteria may contain multiple quorum sensing systems, underlining the importance of intercellular communication. Finally, in some cases different peptides may be recognised by the same receptor, while also hybrid receptors have been constructed that respond to new peptides or show novel responses. This chapter provides an overview of the characteristics of autoinducing peptide-based quorum sensing systems, their application in various Gram-positive bacteria, and the discovery of new systems in natural and engineered ecosystems.

Introduction

A variety of physiological changes in bacterial populations have been shown to be dependent on specific cell densities and growth phases. This phenomenon of cell density-dependent gene expression has been termed quorum sensing, and was initially found to be involved in the regulation of bioluminescence in *Vibrio fischeri* (25). Since then, many other quorum sensing systems have been discovered in both Gram-negative (103) and Gram-positive bacteria (48). Well-studied examples in Gram-negative bacteria include virulence and biofilm-formation in *Pseudomonas aeruginosa* (87, 104), swarming motility of *Serratia liquefaciens* (22) and root-nodule formation by *Rhizobium leguminosarum* (29). In Gram-positive bacteria quorum sensing regulation includes genetic competence in *Bacillus subtilis* (96) and *Streptococcus pneumoniae* (8), virulence in *Staphylococcus aureus* (42), and the production of antimicrobial peptides, including bacteriocins and lantibiotics, in lactic acid bacteria (49, 70). For regulation of these quorum sensing systems bacteria produce extracellular signalling molecules that are responsible for bacterial cell-to-cell communication. Several distinct families of signalling molecules have been identified so far, including *N*-acyl homoserine lactones, 4-quinolones, di-keto-piperazines, autoinducer-2 and peptides (Fig. 2.1) (38, 92). While many Gram-negative bacteria communicate via *N*-acyl-homoserine lactones (Fig. 2.1A), peptides are the most common and well-studied signalling molecules in Gram-positive bacteria, here referred to as autoinducing peptides (AIP). These signalling peptides show a variety of structures but share a small size, are ribosomally synthesised, and are in some cases subject to post-translational modifications that add to their stability and functionality (Fig. 2.1B). It has been suggested that genes for autoinducing peptides may also be present in Gram-negative bacteria, implying that peptide-based signalling is a general system in bacteria (64).

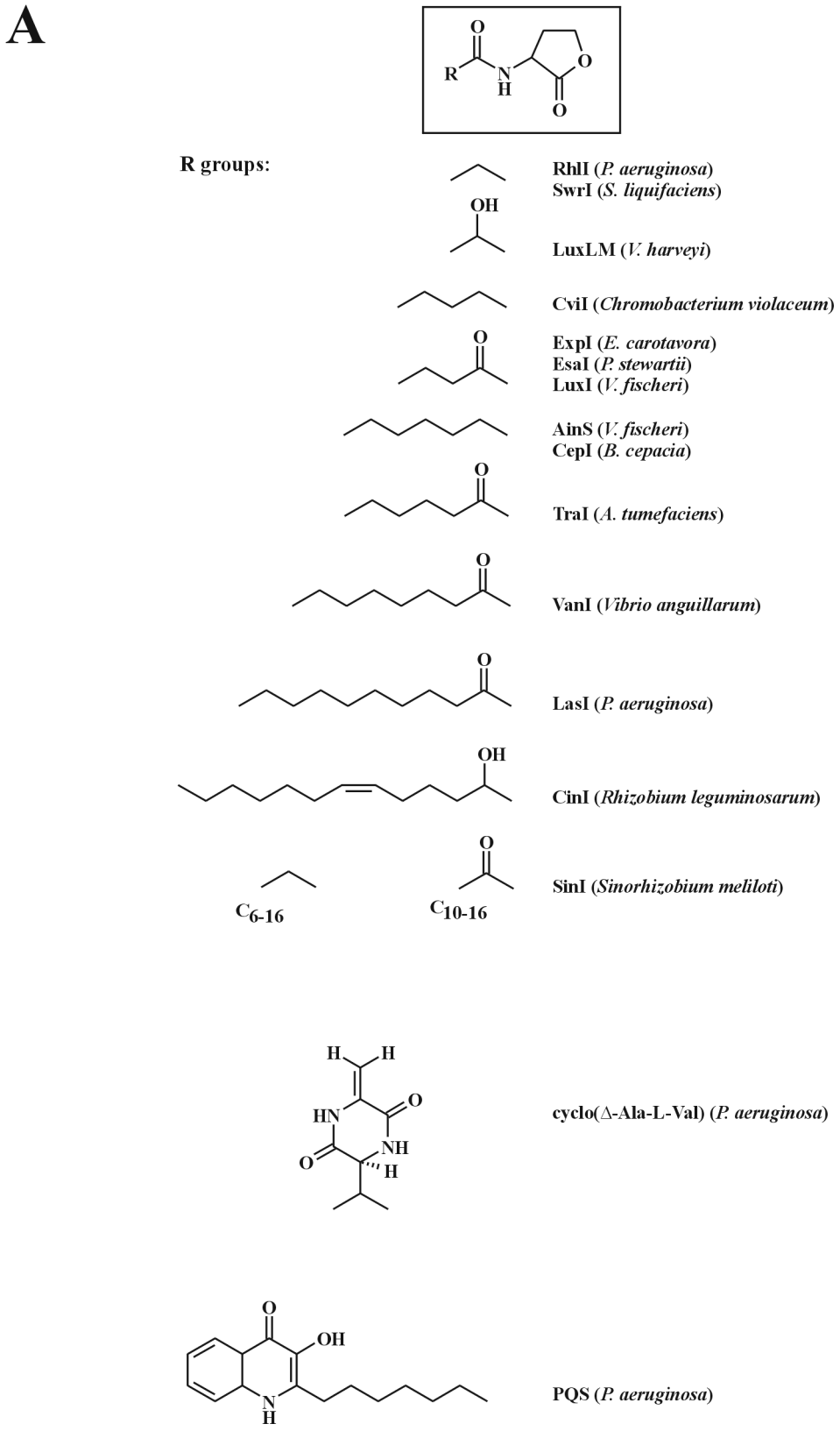

Figure 2.1 (A) Structural diversity of signaling molecules found in Gram-negative bacteria. (*Upper*) *N*-acyl homoserine lactones with the common homoserine lactone moiety (boxed) and different side chains (R groups) shown. (*Lower*) Other autoinducer structures from Gram-negative bacteria. PQS: *Pseudomonas* quinolone signal. Cyclo(Δ-Ala-L-Val): diketopiperazine.

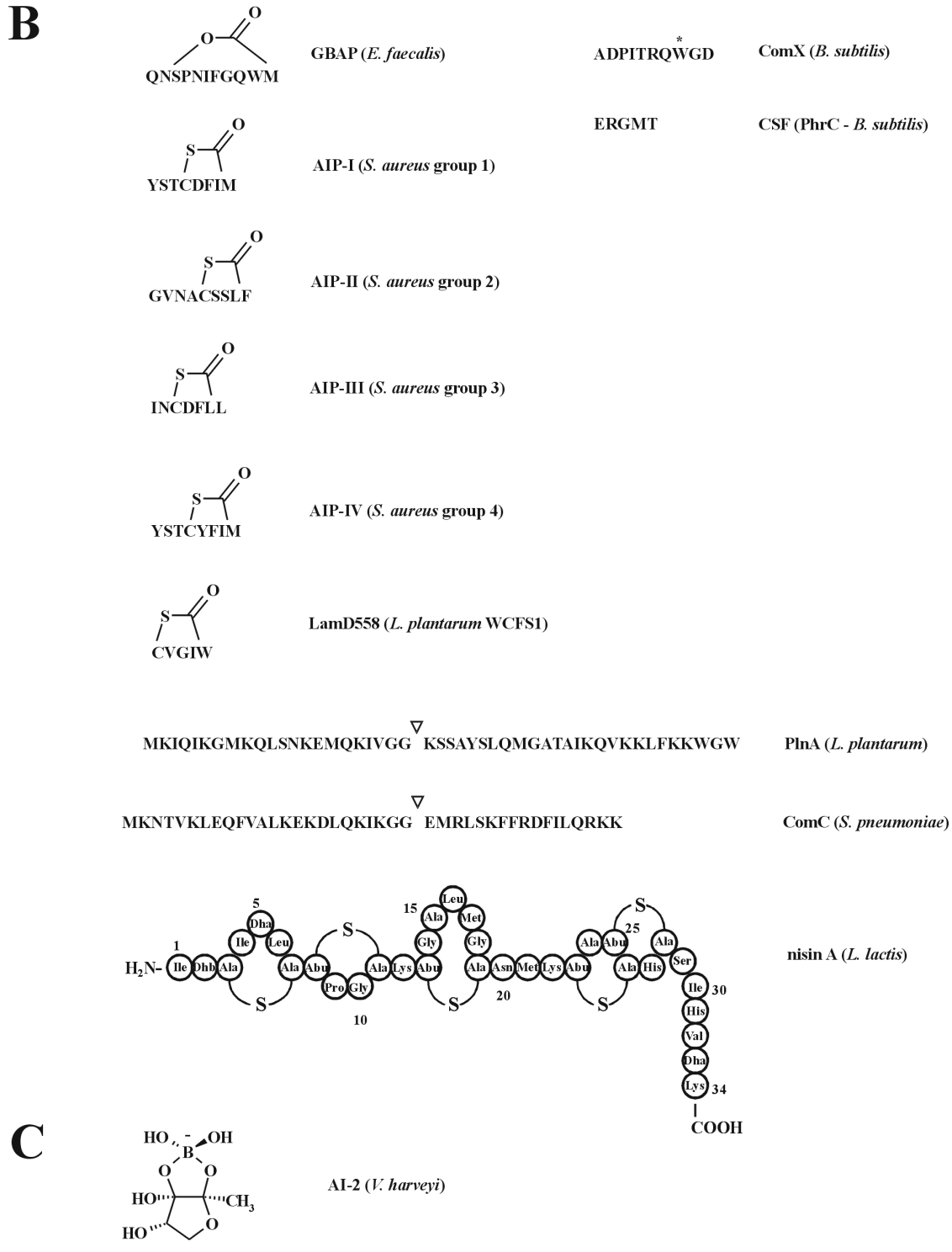

Figure 2.1 (B) Structural diversity of autoinducing peptides found in Gram-positive bacteria. ComX contains an isoprenoid-modification at the tryptophan residue (asterisk). CSF: Competence and Sporulation Factor (PhrC pentapeptide). AIP I-IV: cyclic thiolactone-peptide groups of *Staphylococcus aureus*. Double-glycine-type leader-peptide AIPs involved in regulation of class II AMP production (PlnA of *L. plantarum* C11) and genetic competence (ComC of *Streptococcus pneumoniae*) are shown with the leader peptide. Nisin A: lantibiotic of *Lactococcus lactis*. Ala-S-Ala: lanthionine, Abu-S-Ala: β-methyl-lanthionine, Dha: dehydroalanine, Dhb: dehydrobutyrine. (C) Structure of autoinducer-2 from *Vibrio harveyi*.

Autoinducer-2 (AI-2), which is a furanosyl-borate-diester (Fig. 2.1C), has been detected in both gram-negative and gram-positive bacteria and may therefore serve as an interspecies signalling molecule (7, 106).

Two-component regulatory systems involved in cell-to-cell communication in Gram-positive bacteria

Various ways are known by which peptide-based cell-to-cell communication operates in Gram-positive bacteria (Fig. 2.2), but they all involve one or both elements of a two-component regulatory signal transduction system (TCS), consisting of a membrane-located receptor histidine protein kinase (HPK) and an intracellular response regulator (RR) (30, 76). Autoinducing peptides are ribosomally synthesised as precursor peptides and exported from the cells. At various stages the precursor peptides may be subject to one or more posttranslational modification events, till the active and usually stable autoinducing peptide is produced. In most cases the autoinducing peptide only functions as a signalling molecule, but exceptions include lantibiotics like nisin (52) and subtilin (90) that not only have signalling activity but also show antimicrobial activity (see below). While the autoinduction loop always proceeds via a response regulator that upon phosphorylation activates transcription of target genes as well as genes involved in production of the autoinducing peptides, the mechanism by which the precursor peptides are exported and finally sensed by the cells varies considerably (Fig. 2.2).The simplest quorum sensing systems that were the first to be discovered, involve a dedicated ABC exporter and all elements of a two-component regulatory system (48) (Fig. 2.2A). It is assumed that the autoinducing peptide is produced at a low level during growth and at a certain cell-density reaches a thresh-hold concentration, upon which activation of the receptor kinase takes place. The receptor kinase is then auto-phosphorylated, and subsequently phosphorylates the response regulator that transcriptionally activates the structural gene for the autoinducer, the genes for the two-component regulatory system, and often also the ABC exporter genes, resulting in autoinduction in a dynamic range. This simple quorum sensing system has first been described in *Lactococcus lactis* and *Streptococcus pneumoniae* (35, 52). Subsequently, it was found in many other genera, including *Bacillus*, *Carnobacterium*, *Enterococcus,* and *Lactobacillus* spp. (18, 49, 71, 82). In addition to induction of the regulatory genes, many other genes are

transcriptionally activated by the quorum sensing system, such as those involved in bacteriocin-production, competence-development, or modification of the autoinducing peptides (for reviews see (48, 49, 70).

A modification of the general peptide-based quorum sensing system is that found for the *agr*- and *fsr*-systems in *Staphylococcus aureus* and *Enterococcus faecalis* respectively (43, 67). The *agr*-system in staphylococci encodes a receptor kinase AgrC, a response regulator AgrA, an autoinducing peptide derived from prepeptide AgrD and additionally AgrB, a protein that is involved in processing and posttranslational modification of AgrD (110) (Fig. 2.2B). The corresponding regulatory proteins and a fused FsrBD protein can also be found in the *fsr*-system (see below) (Fig. 2.2C). Another difference is the nature of the peptide export-system, which is not an ABC transporter but a dedicated protein, which has been suggested to be AgrB or FsrB in staphylococci and *E. faecalis* respectively (67, 110). In all cases, the genes involved in the production, modification and export of the autoinducing peptide as well as those coding for the TCS are induced. Moreover, various other genes are induced and these include known virulence determinants in *S. aureus* or those implied in virulence such as the *E. faecalis* gelatinase and serine protease (Figs. 2.2B and C).

Another type of peptide-mediated regulatory system is found for the *phr* self-signalling system in *Bacillus subtilis*, which is involved in regulation of sporulation and genetic competence (63, 79) (Fig. 2.2D). Phr precursor pro-peptides (38-44 amino acids) are exported and cleaved by SecA-dependent systems, and the resulting pro-peptides (11-19 amino acids) are extracellularly processed to the active pentapeptides, which are subsequently imported by an oligopeptide transport-system (Opp) (44). Phr-pentapeptides inactivate Rap-phosphatases that dephosphorylate response regulators of two-component regulatory systems. The production of the *phr* peptides may result in an activation of their own production and this represents the autoregulation loop that is characteristic of quorum sensing systems. Extracellular signalling peptides that act intracellularly have also been identified in *Enterococcus faecalis* as mating pheromones that regulate plasmid transfer (9).

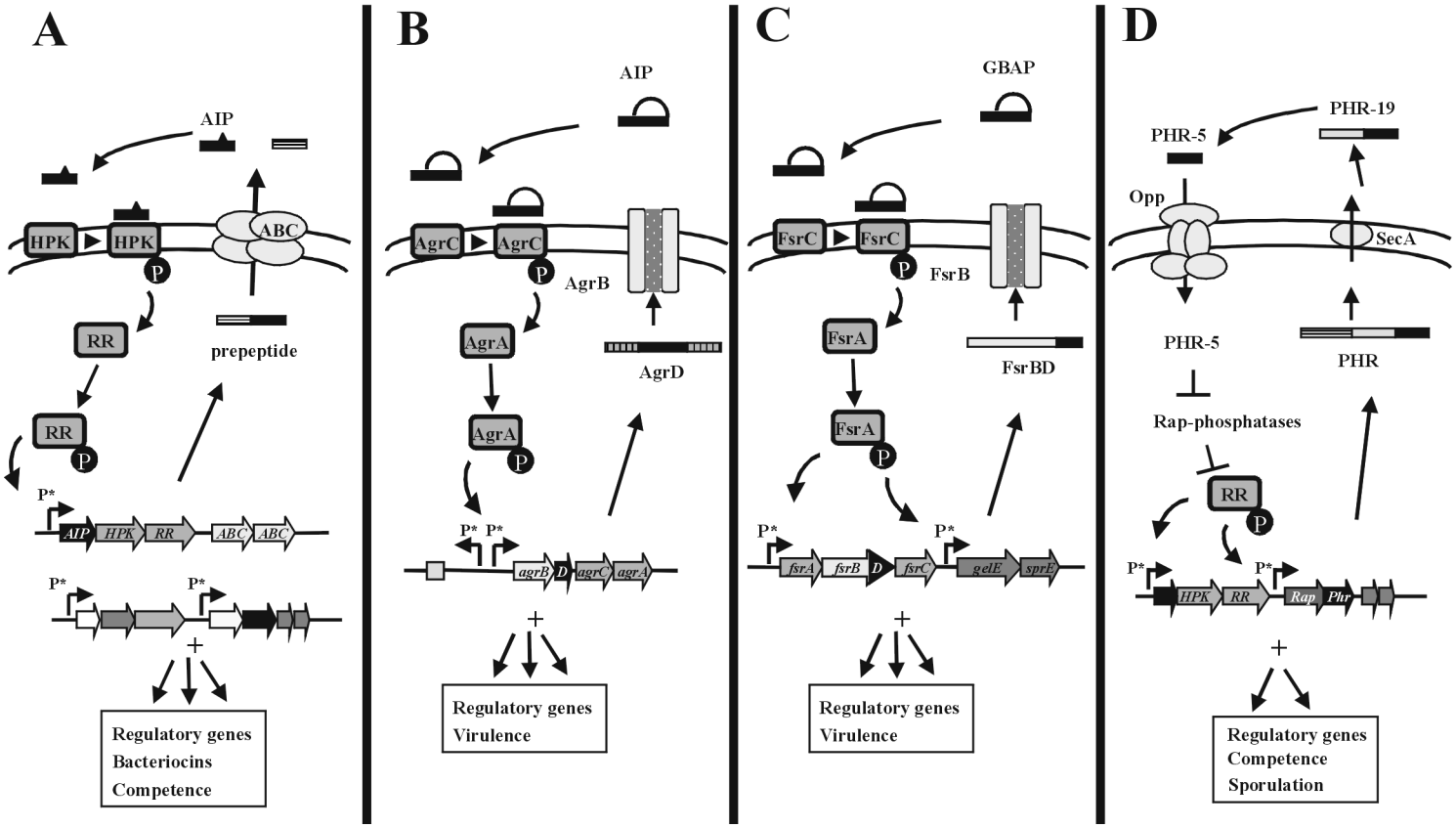

Figure 2.2 Models of peptide-mediated regulatory systems found in Gram-positive bacteria. P* indicates regulated promoters in each system (A) General system for Gram-positive bacteria. HPK: histidine protein kinase; RR: response regulator; ABC: ABC transporter-system; P: phosphate. (B) Staphylococcal *agr*-system. AgrA: response regulator; AgrB: putative AIP-processing/ transporter protein; AgrC: histidine protein kinase; AgrD: AIP prepeptide (C) *Enterococcus faecalis fsr*-system. FsrA: response regulator; FsrB: putative GBAP-processing/ transporter protein; FsrBD: FsrB-GBAP fusion protein; FsrC: histidine protein kinase; GBAP: gelatinase biosynthesis-activating pheromone; *gelE*: gelatinase gene; *sprE*: serine protease gene. (D) Phr-peptide regulatory system of *Bacillus subtilis*. Opp: oligopeptide-permease; SecA: SecA-dependent transport system; PHR: Phr precursor pro-peptide; PHR-19: Phr-pro-peptide; PHR-5: Phr-pentapeptide; Rap: Rap-phosphatase.

Processing and modification of autoinducing peptides

Autoinducing peptides are processed during export from the cell and in several cases subject to posttranslational modification before or after export. Lantibiotics, like nisin in *Lactococcus lactis* or subtilin in *Bacillus subtilis* are class I antimicrobial peptides that contain modifications such as dehydrated amino acids and typical (β-methyl)-lanthionines (see Fig. 2.1B). The modification reactions occur intracellularly on the precursor peptide and involve several modification enzymes, which are all encoded by genes that are also subject to autoregulation (16, 61). The modified precursor peptides are exported by a dedicated ABC exporter and activated by proteolytic removal of the leader sequence (50, 99). In several cases this is catalysed by a specific leader peptidase, encoded by a gene that is autoregulated too (48, 61). Autoinducing peptides involved in the production of class II antimicrobial peptides (bacteriocins) and genetic competence are linear peptides (Fig. 2.1B). These are not known to be modified but contain a typical double-glycine-type leader peptide. This leader peptide is cleaved from the precursor peptide during export by a dedicated ABC-transporter system, encoded by a gene that is autoregulated too (36, 70). For this function the ABC exporter has an N-terminal extension that contains a characteristic Peptidase C39 protease-family domain (Pfam accession number PF03412) (3). This typical processing of AIPs is widely spread and has been reported for the competence-inducing peptide ComC in *Streptococcus pneumoniae* (36), and bacteriocin-inducing peptides in lactic acid bacteria (49, 70).

A specific type of modification is found in the autoinducing peptides that are involved in the *agr*- and *fsr*-systems in *S. aureus* and *E. faecalis,* where the peptides contain respectively a cyclic thiolactone or cyclic lactone structure (Figure 2.1B). Here the actual AIP is cleaved off as an internal part from the prepeptide and then modified, probably during export. In *S. aureus* this is thought to be catalysed via integration of the N-terminal region of the prepeptide into the cytoplasmic membrane, after which the transmembrane protein AgrB can further process and export it via an unknown mechanism (110, 112) (Figs. 2B and C). Remarkably, it has been found that the FsrB protein is in fact a fusion protein consisting of an N-terminal exporter-domain and a C-terminal extension including the autoinducing peptide, gelatinase biosynthesis-activating pheromone (GBAP), which is cleaved off (67). Recent studies by Nakayama et al. (69) have proposed a GBAP processing mechanism by the FsrB protein, that involves a histidine and cysteine residue that are found to be conserved in all known

AgrB-homologues. These residues are essential for processing by FsrB and might also serve this function in staphylococci (111) and *L. plantarum* WCFS1. Additionally, studies have shown that group-specific processing of AgrD is determined by different segments of the AgrB protein (111). Finally, the ComX pheromone that is involved in competence development in *Bacillus subtilis* (59) contains an isoprenoid modification on a tryptophan residue, which is probably added by protein ComQ (1, 2).

Diversity and specificity of cell-to-cell communication systems

The regulatory genes involved in quorum sensing show high genetic polymorphism in between species and even strains. The highest sequence diversity is found in the N-terminal and linker part of the histidine protein kinase, and in the autoinducing peptide and peptide-processing genes. Diversity and specificity has been well studied for the *agr*-system in *S. aureus* strains (20, 40), and the competence-systems in *B. subtilis* (97) and *Streptococcus* spp. (37, 102). For these systems the inducing activity of each AIP appeared to be specific towards its cognate receptor, indicating the existence of distinct pherotypes. However, in some cases the signalling molecule is not only recognised by its cognate species, but also acts on different strains of the same or related species. This intra- and interspecies cross-talk can be either inhibitory or inducing. In staphylococci to date four AIP pherotypes have been found in *S. aureus* isolates, while non-*S. aureus* AIPs have been detected in many other staphylococcal species (20, 40, 72). Autoinducing peptides produced by one *S. aureus* strain were able to inhibit *agr*-expression in some of the other strains. In addition, cross-inhibition between *S. aureus* strains and *S. epidermidis* or *S. lugdunensis* was detected (43, 74). Specific activation or inhibition of *agr*-expression was clearly shown to be dependent on both amino acid sequence and stereo-chemical structure of the staphylococcal AIP, with a cyclic peptide-structure in all cases being essential for biological activity. For specific self-activation it seems furthermore important that the AIP contains a cyclic thiolactone linkage, although activation by lactam-analogs of these AIPs was also found in some cases, albeit at very high concentrations. Cross-group inhibiting peptides should contain a cyclic structure with either a thiolactone, lactone or lactam linkage (56, 60). Further experiments have shown that activating and inhibiting AIPs interact with the AIP receptor at overlapping binding sites, although the exact mechanisms still have to be determined (57). Cross-induction but not cross-inhibition was also observed for ComX-peptides from *B. subtilis* and *B. mojavensis*

strains (97), the ComC-peptides in *Streptococcus pneumoniae* isolates (102) and the SalA lantibiotic peptides in *Streptococcus salivarius* and *Streptococcus pyogenes* (98).

Some autoinducing peptides can have a dual function: the AIP may have both signalling and antimicrobial activity. The most extensively studied examples of this are the lantibiotics nisin from *Lactococcus lactis* and subtilin from *B. subtilis*, which act as signalling molecules that induce their own biosynthesis (49, 52). In addition, the linear unmodified peptide CbnB2 in *Carnobacterium piscicola* LV17B has been found to be both a bacteriocin and an autoinducing peptide (82). The nisin molecule mediates its induction capacity and antimicrobial activity via different mechanisms. The induction capacity is determined by interactions of specific amino acid residues with the N-terminal domain of the sensor protein, while the antimicrobial activity depends on general structural properties, that cause pore-formation in the cytoplasmic membrane (26, 52, 100).

Cell-to-cell communication in microbial ecosystems

Regulation of physiological responses by quorum sensing could ensure co-ordinated gene expression in a bacterial population. High cell-density ecosystems with many microbial interactions, where there is also a diffusion barrier, could be selective for the development of these quorum sensing systems in bacteria. Co-ordinated responses might lead to competitive advantages for the population, efficient adaptation and responses to changing environmental conditions, or co-ordination of interactions between bacteria and their abiotic or biotic environment. This biological rationale for quorum sensing regulation has been nicely demonstrated for the *in vivo* regulation of staphylococcal virulence factor expression (105). In addition, the secretion of degradative enzymes or other secreted compounds involved in nutrient uptake (such as surfactants and siderophores) will provide a competitive advantage for a single cell if diffusion and mixing in its microenvironment are limited. Hence it has been proposed that quorum sensing systems may also have evolved to determine if diffusion takes places (83).

Cross-talk by autoinducing peptides between strains and species could play an important role in the way microbial ecosystems develop and function. Cross-inhibition of other bacteria is likely to be advantageous to a species during colonisation of abiotic or biotic surfaces such as plant leaves, skin or mucosal epithelium in the gastrointestinal tract (93), or competition for substrates in e.g. dairy products. In

general however, specificity of autoinducing peptides towards their cognate receptors should avoid too much cross-interference. Peptide-induced bacteriocin-production is a phenomenon involved in competition and has been extensively studied in lactic acid bacteria that are competing with other bacteria (23, 61). By producing these natural inhibitors, growth of competing micro-organisms is controlled, and in this way the bacteriocin-producing species may have an advantage in colonisation of ecological niches or access to nutrients. Cross-talk could also lead to co-ordinated co-metabolism among bacterial species or interference with host responses. An interesting example of functional co-ordination between bacteria and their host is found for the human commensal *Bacteroides thetaiotaomicron*. The *Bacteroides thetaiotaomicron* genome encodes 33 novel (hybrid) TCS that are mostly positioned next to sugar utilisation genes and therefore might be involved in coordinating polysaccharide utilisation through interactions with the host (107, 108). Such interactions have already been shown for the FucR transcriptional regulator that regulates expression of fucose utilisation genes and coordinates production of host fucosylated glycans through signalling to the host (39). Production of the lantibiotic ruminococcin A (RumA) in the human commensal *Ruminococcus gnavus* FRE1 has been shown to be dependent on the presence of trypsin while it was shown to be resistant to other digestive proteases. Trypsin could therefore be involved in processing of a putative extracellular AIP, which might act as the inducing signal for the regulatory TCS involved in RumA production. Such activation could be a specific adaptation to the intestinal environment, in which this strain naturally resides (12, 27). Finally the quorum sensing system involved in expression of virulence genes in enterohemorrhagic *E. coli* (EHEC) could be activated by the bacterial autoinducer-3 (AI-3), whose synthesis depends on *luxS*, but also by the human hormone epinephrine. This indicates a novel mechanism of host-microbe inter-kingdom cross-talk (88).

Although many quorum sensing systems have been discovered and studied by now in Gram-positive bacteria, little research has been done on the presence and functionality of these systems in natural microbial ecosystems. Indications of a widespread distribution of quorum sensing in ecosystems comes from the discovery of e.g. *N*-acyl homoserine lactones and autoinducer-2 activity in the rumen (24, 65), but also peptide-controlled class IIa bacteriocin production in human gastrointestinal LAB such as lactobacilli and enterococci (70) and ruminal bacteria like *Butyrivibrio fibrisolvens* (45, 46). Several recent studies have addressed the presence, expression

and function of peptide-based communication systems in microbial communities. For this purpose, molecular approaches have been developed to detect novel or known quorum sensing genes. Oligonucleotide probes based on the amino acid sequence of purified autoinducing peptides or bacteriocins have been used to detect genomic DNA-fragments carrying the genes involved in their biosynthesis, such as for the ruminococcin A regulatory gene cluster (12, 27). Degenerate Oligonucleotide Primer PCR (DOP-PCR) is another powerful method to detect quorum sensing systems and has been applied with degenerate primers that are designed based on the amino acid similarity of quorum sensing two-component systems, notably the conserved regions in the histidine protein kinases. Various potential quorum sensing regulatory genes were discovered by DOP-PCR in several Gram-positive bacteria (66), *Lactococcus lactis* (73) and other lactic acid bacteria (68). Moreover, this approach was also used to detect these systems in the complex ecosystem of the human gastro-intestinal tract (J. Nakayama and M. Sturme, unpublished observations). Another indication for the functionality of quorum-sensing regulated systems in microbial ecosystems comes from the competence systems in *Bacillus* and *Streptococcus* spp., which enables these bacteria to take up free DNA from the environment and to integrate parts of this foreign DNA into their genomes. This can lead to new features in these bacteria, and thereby to increased adaptation to the environment or competitive advantages, or it could have a function in DNA repair mechanisms. Induction of these natural transformation systems at high cell densities increases the probability of naked DNA being available, as free DNA is continuously being produced by lysis of bacterial cells and excretion. Indications for such transformations in natural ecosystems originated from several soil microcosm studies on *Bacillus subtilis* strains (55). The specificity of the quorum sensing-regulated competence system involved might enhance the probability of taking up DNA from closely related strains, and thus reducing the risk of gene disruption and incorporation of toxic genes (96).

Finally, the construction of environmental metagenome libraries is promising in detecting novel quorum sensing systems. Especially the so-called bacterial artificial chromosome (BAC) libraries are gaining more interest, as large genomic fragments (usually 50-150 kb) can be cloned from DNA of entire microbial ecosystems (84). BAC libraries could provide a useful tool for studying genetic and functional diversity of quorum sensing systems in bacterial communities, of which a large part cannot been

cultured yet. Some of the promising applications of BAC libraries have already been described in studies on soil and marine microbial communities (4, 58, 84).

Peptide-based cell-to-cell communication and biofilms

Bacteria are able to adhere to surfaces or each other in biofilms, in which dense cell aggregates are embedded in a matrix of exopolysaccharides (91). Growth in biofilms has been thought to be ecologically advantageous, by providing protection against dehydration or antibiotics, increasing nutrient availability and metabolic cooperativity, or enhancing gene transfer (13, 41). Biofilms are commonly formed by pathogens such as staphylococci that grow on catheters and other polymeric medical devices (28) or *Pseudomonas aeruginosa* that infects lungs of human cystic fibrosis patients (87), but can also be found in complex microbial ecosystems such as dental plaques in the oral cavity (51) or in the gut mucus lining of the gastrointestinal tract (75). The first stage of biofilm formation is initiated by attachment to surfaces, which is dependent on cell-surface interactions as well as environmental conditions, specifically osmolarity (89). Additionally, attachment in staphylococci is probably mediated by cell wall-associated adhesins, including microbial surface components recognizing adhesive matrix molecules (28). The second stage is the formation of microcolonies, which are communities of bacterial cells of three to five layers deep. In staphylococci this is mediated by the polysaccharide intercellular adhesin (PIA), which is influenced by many environmental factors, such as anaerobiosis, iron limitation, high osmolarity and high temperature (28). In *B. subtilis* this is regulated via the response regulator Spo0A that is activated upon starvation (32). Spo0A represses the negative regulator of biofilm formation AbrB, which results in de-repression of production of secreted proteins involved in biofilm formation (33). The third and final stage is the formation of mature biofilm structures that are embedded in an extracellular polymeric matrix. This stage in many cases involves quorum sensing mechanisms (77). Examples of peptide-based quorum sensing systems that are involved in biofilm-formation in gram-positive bacteria include the staphylococcal *agr*-system (101, 109) and the *E. faecalis fsr*-system (34), both involving cyclic (thio-)lactone signalling peptides, and the streptococcal *comCDE*-system, involving the linear, unmodified competence-stimulating peptide (CSP) (54, 80). Remarkably, for *S. aureus* it was found that disruption of the *agr*-system under flow conditions had no

effect, while under static conditions it either inhibited or enhanced biofilm formation (109). These studies indicated that biofilm formation, in staphylococci at least, requires both environmental as well as quorum sensing signals.

Impact of genomics on quorum sensing studies

With the increasing availability of completed genome sequences, genomic data-mining can be used for analysing bacterial genomes and detecting potential quorum sensing systems. These can be analysed in detail for their functionality by mutation and transcriptional profiling studies that may reveal genome-wide effects of autoinducing peptides. An example of this is shown for the completed *S. pneumoniae* genome, which encodes 13 putative TCS (94). Mutational analysis of these TCS revealed several growth-phase regulated TCS, of which at least one was involved in a peptide-dependent cell-to-cell signalling (53, 95). This TCS belonged to the HPK_{10}-subfamily and was part of the *blp* operon (bacteriocin-like peptide). The *blp* operon encoded for a signalling peptide, response regulator, histidine protein kinase, and peptide-transporters. Microarray analysis of expression profiles in *S. pneumoniae* revealed several genes that are regulated by this bacteriocin-like autoinducing peptide (15). Most of them were located near the *blp* operon, suggesting a functional link, and appeared to be cell density-dependent regulated. Microarray analysis of the *B. subtilis* competence quorum sensing-system showed differences between cells expressing ComK (competence transcription factor) and vegetatively growing cells. In addition to the known competence genes, also ComK-regulation of many unsuspected genes was detected (5). Similarly, transcription profiling of genes regulated by the *agr* and *sarA* systems in *S. aureus* revealed the regulation of non-characterised putative virulence factors next to the expected virulence genes, and additionally regulation of more general non-virulence genes (21). Microarray studies therefore appear to be valuable for identifying quorum sensing-regulated genes and elucidating the regulatory mechanisms involved. Genome data mining, functional and comparative genomics are now rapidly evolving for (commensal) lactic acid bacteria too, showing insight into metabolic and regulatory networks, including quorum sensing regulated characteristics (19, 86).

Concluding remarks and perspectives

Until now research on quorum sensing has mainly focused on the autoinducing signals and the regulatory systems involved. These studies have revealed many new quorum sensing systems in individual species and have led to a wealth of sequence information of their genes and regulation. Our current knowledge indicates that cell-to-cell communication by autoinducing peptides plays an important role in regulating cell-density-dependent phenotypes in Gram-positive bacteria, and involves two-component regulatory systems. The autoinducing peptides showed high specificity and diversity, which is reflected in the differences in primary structure as well as in the extent of posttranslational modification.

The advanced knowledge of the regulation and functions of peptide-based quorum sensing systems may lead to the development of new tools in molecular biology and applications in medicine. Quorum sensing-regulated systems could be useful for designing controlled gene-expression systems in Gram-positive bacteria, as can be illustrated by the food-grade nisin-controlled expression system (NICE), which is based on the autoregulatory nisin gene cluster from *L. lactis* (14, 17). It is a transferable system consisting of a regulatory plasmid carrying the *nisRK* regulatory genes and an expression vector with the cloned gene of interest under control of the *nisA* promoter. Successful implementation of the NICE-system was demonstrated in various lactic acid and other low-GC Gram-positive bacteria such as: *Lactococcus lactis*, *Leuconostoc lactis*, *Lactobacillus plantarum, Enterococcus faecalis, Streptococcus thermophilus, Bacillus subtilis,* and *Staphylococcus aureus* (6, 47, 78). Insight into the effects of peptide-structure on the activity of autoinducing peptides might lead to the rational design of synthetic peptides that are able to inhibit quorum sensing-regulated phenotypes. The potential of such a strategy has already been shown in studies on quorum sensing-blocking synthetic peptides that were designed based on knowledge of *S. aureus*-AIP structure-function relationships (56, 62, 85). In other studies concerning targeted inhibition of virulence and infections caused by *S. aureus*, a chimeric peptide of an AgrD-peptide fused to a bacteriocin was shown to specifically kill *S. aureus* cells, but not the related *Staphyloccus epidermidis* or unrelated *Streptococcus pneumoniae* (81). Similar approaches that target quorum sensing signals may also be applied to the quorum sensing systems involved in the production of

virulence factors in *Enterococcus faecalis*, a main cause of hospital infections for which few therapeutics exist (10, 11, 31, 67).

The rapid increase in sequence information and recent insights into regulation of quorum sensing genes can now be applied to investigate the genes that are regulated by these systems (regulons). Genome-wide approaches such as those based on transcriptome or proteome analysis can impact substantially on this area by revealing complex global regulatory mechanisms, and this knowledge may ultimately be used to study the *in situ* activity and the role of quorum sensing systems in natural ecosystems.

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Genomic analysis of quorum sensing systems in *Lactobacillus plantarum* WCFS1

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Abstract

The *Lactobacillus plantarum* genome was predicted to encode 13 genetically linked two-component regulatory systems (TCS) consisting of a histidine protein kinase (HPK) and response regulator (RR) gene. An orphan HPK and orphan RR that were located at different positions on the genome might encode a fourteenth TCS. Four of the TCS showed significant similarities to those involved in peptide-based quorum sensing, and these were analyzed using bioinformatics and functional genomics approaches. Three TCS contained an upstream gene encoding a putative autoinducing peptide (AIP), of which two were preceded by a double-glycine-type leader-peptide. One of these was identical to the *plnABCD* regulatory system and was shown to functionally regulate plantaricin production in *L. plantarum* strain WCFS1. A third TCS showing a similar gene organization as the *Staphylococcus aureus agrBDCA* quorum sensing system, was designated *lamBDCA* for *L*actobacillus *agr*-like module. The *lamD* gene of this system was shown to encode a cyclic thiolactone peptide. A fourth TCS was paralogous to the *lam*-system, but lacked the *lamBD* genes. Finally, the orphan HPK and RR showed clear peptide-based quorum sensing characteristics, and could therefore form a fifth peptide-based quorum sensing TCS. Besides these peptide-based quorum sensing TCS, the *luxS* gene encoding the autoinducer-2 synthase was identified. For some of these predicted quorum sensing systems their functionality could be confirmed experimentally. The relatively high number of predicted peptide-based quorum sensing TCS might reflect the ability of *L. plantarum* to survive in a diverse range of ecological niches.

Introduction

The lactic acid bacteria (LAB) comprise a diverse group of Gram-positive bacteria that are applied worldwide in the production of fermented food products such as dairy, meat and vegetable products (9, 50). Next to this, several LAB are also found in the gastrointestinal tracts of humans and other animals (63). Among the LAB in particular strains from the genus *Lactobacillus*, are considered to exert health-promoting effects in man and animals (46). *Lactobacillus plantarum* is a species that is encountered in a range of environmental niches like fermented food products and on plant material (13, 15). Moreover, *L. plantarum* is a natural inhabitant of the human GI-tract (1, 10) and some *L. plantarum* strains are marketed as probiotics. *L. plantarum* WCFS1 is a single colony isolate of the oesophagal strain *L. plantarum* NCIMB8826 (23), which was shown to survive stomach passage and persist in the human GI-tract after a single dosage (64). To allow for efficient colonization and persistence or effective adaptation to changing conditions in the environment, *L. plantarum* needs sensory systems to detect (specific) environmental signals. In bacteria, this function is commonly mediated by two-component signal transduction systems (TCS) that monitor and respond to changes in environmental conditions such as osmolarity, nutrient availability (C, N, P, metal ions), temperature or pH (14, 25). However, notably in Gram-positive species, TCS are also used to respond to specific secreted signaling molecules in quorum sensing (58).

Quorum sensing TCS in Gram-positive bacteria regulate the expression of genes involved in diverse functions such as virulence, genetic competence, or the production of bacteriocins (43, 45, 62). This modulation is done in a coordinated and cell density-dependent manner. For this, Gram-positive bacteria use specific signaling peptides that are often post-translationally modified and exported by dedicated transport systems, and finally sensed by responsive cells via membrane-located receptors that are part of TCS (58). Bacteria may contain multiple quorum sensing systems, underlining the importance of intercellular communication (32, 58).

We describe here the genomic analysis of TCS that were annotated on the completed *L. plantarum* WCFS1 genome sequence (33), and could be involved in quorum sensing. In addition, the experimental confirmation for the functionality of some of these TCS is discussed.

Methods

Architecture of quorum sensing two-component regulatory systems

Two-component regulatory systems (TCS) consist of a membrane-located sensor protein or histidine protein kinase (HPK), which monitors one or more environmental factors, and a cytoplasmic effector protein or response regulator (RR), which modulates expression of specific genes. The genes encoding these canonical proteins in general are encoded in an operon structure consisting of the cognate HPK and RR. Signal transduction proceeds via the HPK, which upon signal recognition autophosphorylates a conserved histidine residue. Subsequently, phosphotransfer to a conserved aspartate residue in the cognate RR takes place, which consecutively becomes activated as a transcriptional regulator (25) (see also Fig. 3.1). Some HPKs have also been described to act as phosphatases under non-inducing conditions, thereby silencing the signal transduction system (26, 31).

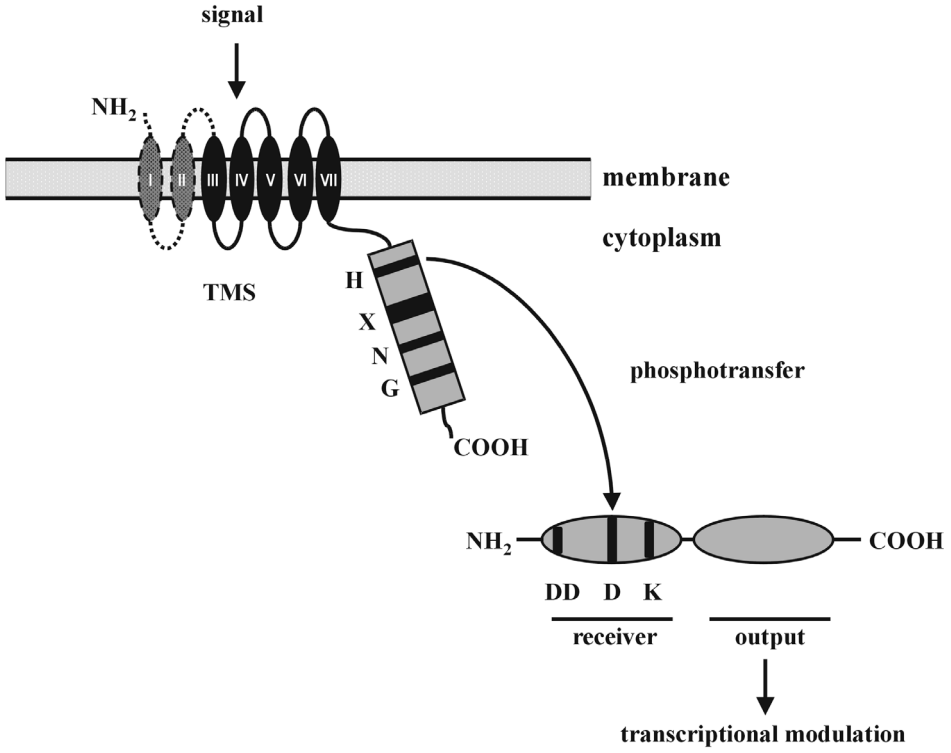


Figure 3.1 General signal transduction mechanism of two-component regulatory systems. The cellular localization and sequence motifs characteristic of two-component histidine protein kinases of the HPK_{10}-subfamily and response regulators are depicted (see also Table 1). TMS: Trans-Membrane Segments I-VII. Homology boxes of the HPK_{10}-subfamily as described in the text are: H, X, N, and G. Conserved residues in the N-terminal CheY-like receiver domain of response regulators as described in the text are: DD, D, and K.

HPKs contain a conserved C-terminal ATP-binding site in which the phospho-accepting histidine residue is located as well as highly conserved clusters of residues, the so called homology boxes (20, 48). A comprehensive classification of HPKs, based on the presence and structure of the various homology boxes, was made by Grebe and Stock (20). In this classification, the HPKs of various peptide-based quorum sensing TCS (QS-TCS) comprise a specific sub-family called HPK_{10}. The sub-family is small and contains e.g. AgrC and FsrC of staphylococci and enterococci, respectively, ComD of streptococci, and in addition HPKs related to bacteriocin production in lactic acid bacteria (43). Nevertheless, these homology-boxes are not universally conserved in all peptide-based QS-TCS, since they are absent in the peptide-sensing competence regulating HPK (ComP) of *Bacillus subtilis* (51, 68), which belongs to the HPK_7-subfamily (20). The homology boxes that are present in the HPK_{10}-subfamily show the following characteristics: the H-box (site of histidine phosphorylation) has a characteristic tyrosine two residues downstream from the conserved histidine (characteristic motif F+HDY.N) and no conserved proline residue at position 5 downstream; the X-box (conserved hydrophobicity pattern) is present; the N-box has only one conserved asparagine residue (characteristic motif DNAIE); and the G-box (plays critical role in phosphotransfer) has a characteristic STKG..RGhGL motif (see Fig. 3.1). The D-box that normally is part of the nucleotide-binding domain is absent in this family. Furthermore, the HPK_{10} subfamily members possess 5-7 N-terminal transmembrane segments (TMS) (see Fig. 3.1).

General features of TCS RRs are a C-terminal DNA-binding domain and a N-terminal, CheY-like receiver domain (65) (also called REC-domain: SMART accession number SM00448). The phospho-accepting aspartate (D) is located centrally in the receiver domain, and is flanked by conserved aspartate residues near the amino-terminal part of this domain (usually DD; but ED is also regularly found) and a lysine residue (K) near the carboxy-terminal part of this domain (47, 49) (see Fig. 3.1). These residues all contribute to the acidic pocket of the phosphorylation site. The RRs have been classified too by Grebe and Stock (20) on basis of the receiver domain and on basis of the DNA-binding domain. In both classifications the RRs related to HPKs of the HPK_{10} sub-family comprise a separate sub-family: the RD and the ComE subfamily, respectively. A more recent analysis showed that most RRs of peptide-based QS-TCS belong to the LytTR family of response regulators (PFAM accession number PF04397), based on a conserved motif in the C-terminal helix-turn-helix

(HTH) DNA-binding domain (44). Typical representatives have been identified in AgrA and FsrA of staphylococci and enterococci, respectively, as well as ComE in streptococci, and in RRs involved in bacteriocin production in lactic acid bacteria (43). In analogy to its related HPK, the competence-regulating RR (ComA) of *Bacillus subtilis* (67) does not show the above-mentioned characteristics of RRs belonging to peptide-based QS-TCS, but instead belongs to the RE-subfamily based on its receiver domain (20) and contains a HTH-LuxR DNA-binding domain (17).

For the peptide-based QS-TCS of Gram-positive bacteria, genes encoding the cognate signaling peptide - the so-called autoinducing peptide (AIP) - are generally found upstream of the genes encoding the TCS. For some peptide-based QS-TCS additional genes encoding AIP transporting proteins (often ABC transporters (22)) or modifying proteins are also genetically linked (see for more details refs (32, 58)).

Prediction of QS-TCS in *L. plantarum* WCFS1

Annotation of the completed genome sequence of *L. plantarum* WCFS1 (described in ref (33)) has shown that it codes for at least 13 genetically linked, complete TCS consisting of a HPK and RR gene. In addition, one orphan HPK (*hpk9*: lp_3063) and one orphan RR (*rrp8*: lp_2665) were found. The chromosomal distribution of the *L. plantarum* TCS-encoding genes appeared to be random, although there might be a slight over-representation in the so-called life-style adaptation islands between 2.70 to 2.85 and 3.10 to 3.29 Mb of the WCFS1 chromosome (33, 41) (Fig. 3.2). The number of TCS in *L. plantarum* is in the same range as was found for Gram-positive pathogens with a comparable genome size, such as *Staphylococcus aureus* (35) and *Enterococcus faecalis* (21), each with 17 TCS, or *Listeria monocytogenes* with 15 TCS (18). However, this number is significantly lower then the 36 TCS found in *B. subtilis*, which only has a 1.3-fold larger genome (16). The genomes of the related intestinal LAB species *L. acidophilus* (2) and *L. johnsonii* (8, 52) (both 1.99 Mb), were both predicted to code for 9 complete TCS.

The 14 putative TCS on the *L. plantarum* WCFS1 genome were analyzed to predict which genes could constitute QS-TCS, based on the following criteria (details described above): (i) gene and protein similarity of HPKs and RRs with recognized quorum sensing systems, (ii) genomic linkage between HPKs and RRs, (iii) quorum sensing-specific protein architecture of HPKs and RR (domains, residues and transmembrane regions, i.e. the HPK_{10} and RD/ComE sub-families of ref 19), (iv)

presence of AIP-like genes or additional quorum sensing-related genes. Similarity searches for DNA or protein sequences of the respective genes were performed with BLASTN and BLASTP using default settings (3). Protein-domains were predicted using SMART, including outlier homologues and PFAM domains (http://smart.embl-heidelberg.de/) (36, 57), and transmembrane segments were predicted using SOSUI (24) and TMHMM-2.0 (34).

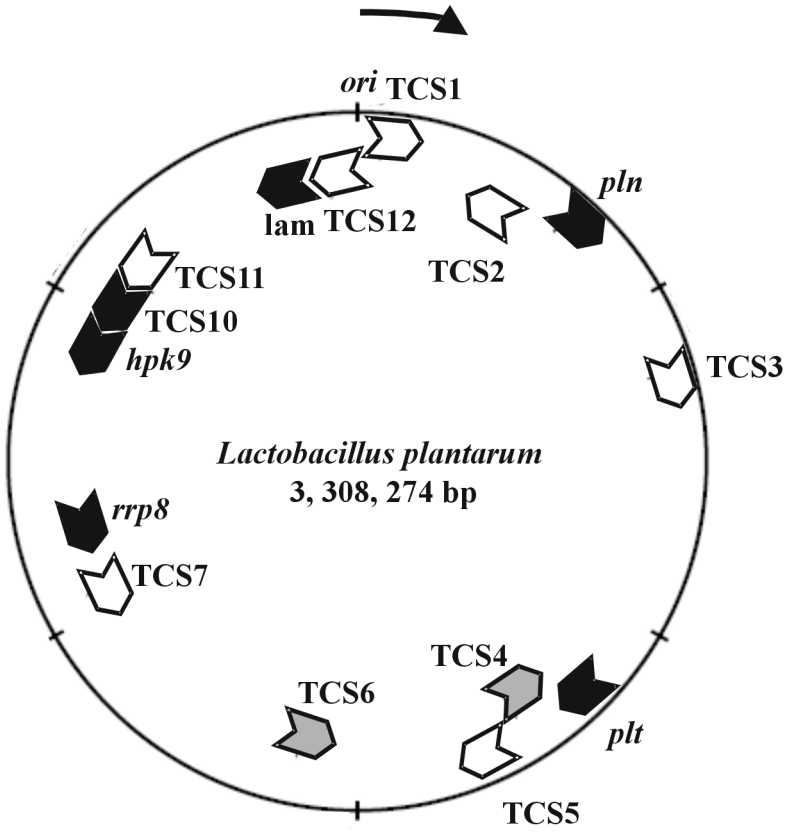


Figure 3.2 Distribution of two-component regulatory systems on the *L. plantarum* WCFS1 genome and their characteristics as described in the text. The outer continuous circle shows the circular genome with marked positions (clockwise orientation); the middle circle shows the TCS on the DNA plus-strand and the inner circle shows the TCS on the DNA minus-strand. Black arrows: TCS with HPKs of the HPK_{10}-subfamily and RRs with LytTR DNA-binding domains. Grey arrows: TCS with RRs with HTH-LuxR DNA-binding domains. White arrows: TCS without characteristic quorum sensing domains in HPK and RR. *ori*: origin of replication.

The HPK_{10}-subfamily of ref (20) encompasses peptide-based quorum sensing HPKs that were experimentally confirmed, and can therefore be used for assigning putative peptide-based quorum sensing functions to HPK genes. For RR genes the presence of a LytTR-domain does not in all cases classify a RR as a quorum sensing gene (44). However, the presence of an adjacent HPK with HPK_{10}-subfamily characteristics can be used to classify the RR as such.

Using the above-mentioned criteria, the *L. plantarum* genome is predicted to encode 5 HPKs that show characteristics of the HPK_{10}-subfamily and 6 RRs that contained a HTH-DNA-binding domain of the LytTR family (44) (Table 3.1). Four pairs of adjacent HPK and RR encoding genes were predicted to constitute complete TCSs that could be involved in peptide-based quorum sensing, based on their amino acid sequence homologies and the presence of specific motifs and domains, as described above. In addition an orphan HPK and RR showing these characteristics were detected. The relevant features of these HPKs and RRs will be discussed below and are summarized in Table 3.1 and visualized in Figure 3.3.

Table 3.1. General features of HPKs and RRs of quorum sensing-like TCS of *L. plantarum* WCFS1 (see also Figure 3.3)

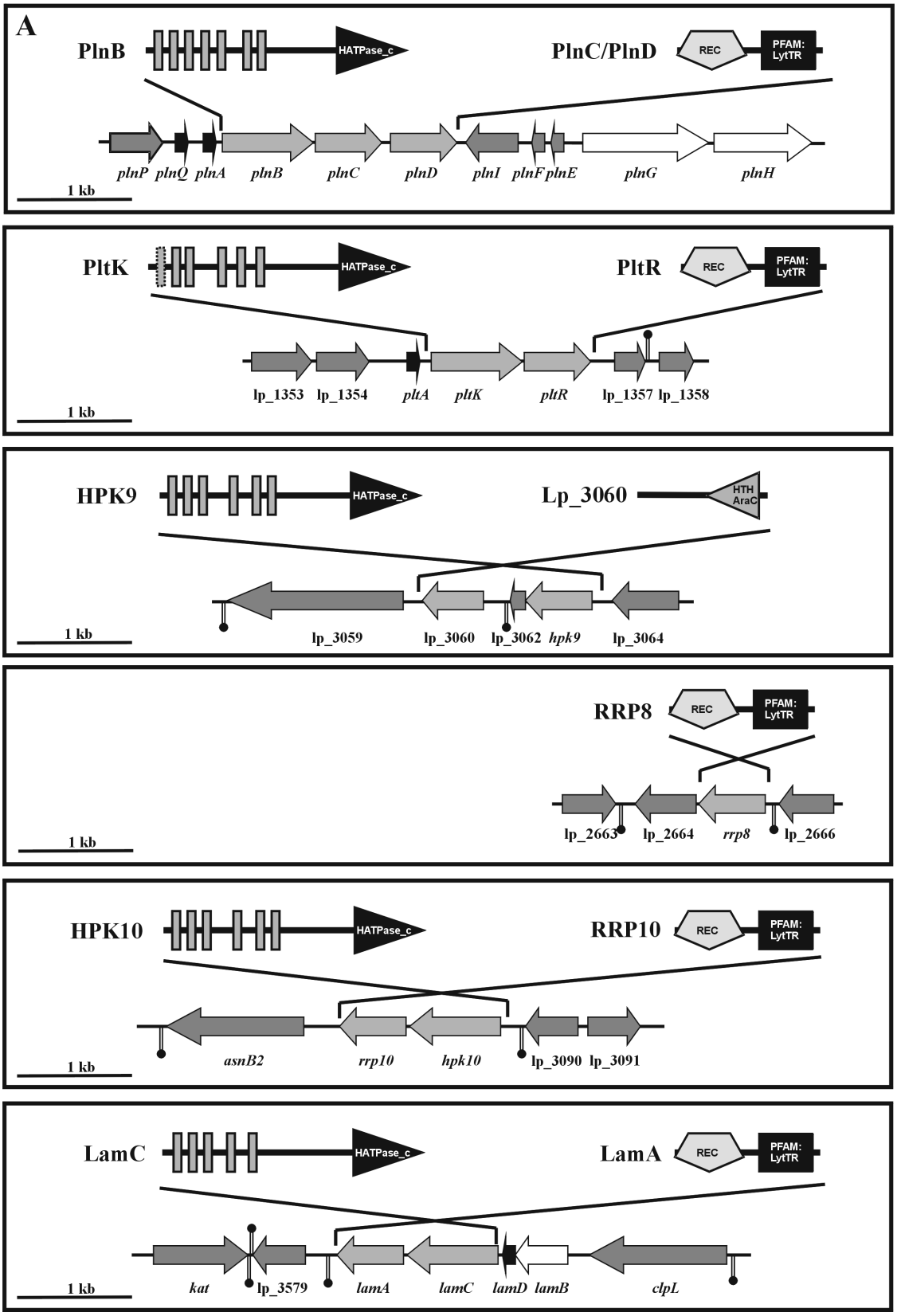
Gene name*	ORF no.†	Size (AA)	TMS‡	HPK -subfamily§	Gene name*	ORF no.†	Size (AA)	DNA-binding domain	AIP	ORF no.†
HPK					RR					
plnB	lp_0416	442	7‖	HPK_{10}	*plnC*	lp_0417	247	LytTR	*plnA*	lp_0415
					plnD	lp_0418	247	LytTR		
pltK	lp_1355	420	5-6	HPK_{10}	*pltR*	lp_1356	255	LytTR	*pltA*	lp_1354a
hpk4	lp_1488	343	-	HPK_7	*rrp4*	lp_1487	217	HTH-LuxR		
hpk6	lp_1943	367	4-5	HPK_7	*rrp6*	lp_1942	201	HTH-LuxR		
					rrp8	lp_2665	249	LytTR		
hpk9	lp_3063	422	6	HPK_{10}		lp_3060	253	HTH-AraC		
hpk10	lp_3088	416	6	HPK_{10}	*rrp10*	lp_3087	248	LytTR		
lamC	lp_3581	419	5‖	HPK_{10}	*lamA*	lp_3580	247	LytTR	*lamD*	lp_3581a

*,† Gene name and lp_number from the *L. plantarum* WCFS1 genome website (http://www.cmbi.kun.nl/lactobacillus).

‡ TMS: Trans Membrane Segments as predicted from TMHMM-2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html).

§ HPK classification after Grebe and Stock (20).

‖ Experimentally confirmed data for PlnB in *L. plantarum* C11 (28) and AgrC in *S. aureus* (37).


A
PlnB
HATPase_c
PlnC/PlnD
REC
PFAM: LytTR
plnP plnQ plnA plnB plnC plnD plnI plnF plnE plnG plnH
1 kb
PltK
HATPase_c
PltR
REC
PFAM: LytTR
lp_1353 lp_1354 pltA pltK pltR lp_1357 lp_1358
1 kb
HPK9
HATPase_c
Lp_3060
HTH AraC
lp_3059 lp_3060 lp_3062 hpk9 lp_3064
1 kb
RRP8
REC
PFAM: LytTR
lp_2663 lp_2664 rrp8 lp_2666
1 kb
HPK10
HATPase_c
RRP10
REC
PFAM: LytTR
asnB2 rrp10 hpk10 lp_3090 lp_3091
1 kb
LamC
HATPase_c
LamA
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PFAM: LytTR
kat lp_3579 lamA lamC lamD lamB clpL
1 kb

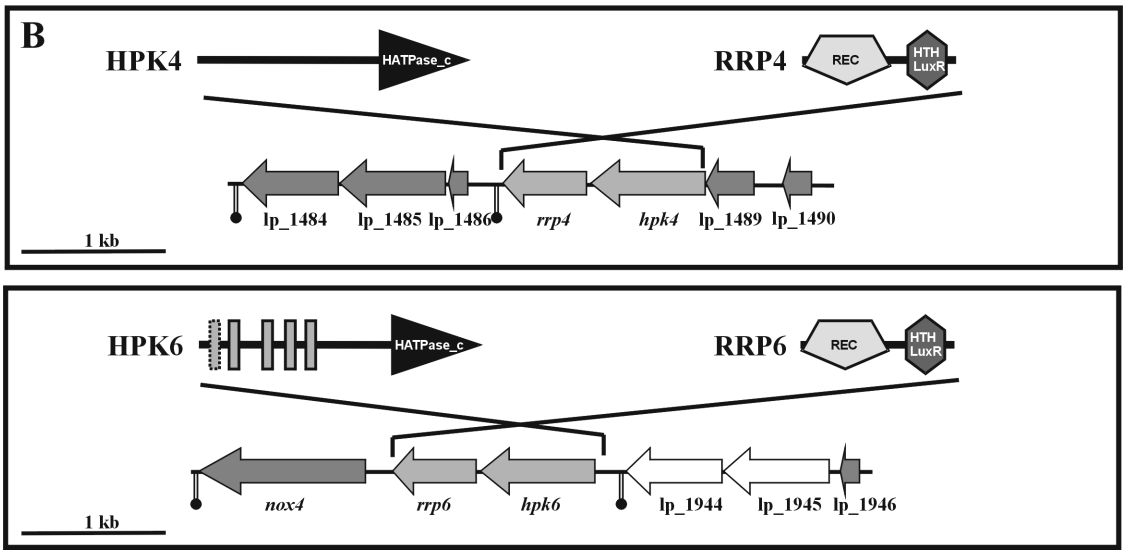


Figure 3.3 Architecture and genomic organization of two-component histidine protein kinases and response regulators that contain quorum-sensing associated domains, according to SMART-descriptions (http://smart.embl-heidelberg.de/smart) or PFAM-families (http://www.sanger.ac.uk/Software/Pfam). (A) HPK_{10}-subfamily type HPKs and LytTR-family type RRs. (B) HPK_7-subfamily type HPKs and LuxR-family type RRs. HATPase: histidine-kinase-like ATPase-domain; REC: cheY-homologous receiver domain; PFAM LytTR: LytTR DNA-binding domain; HTH-LuxR: helix-turn-helix Lux regulon; HTH-AraC: helix-turn-helix arabinose operon control protein. Rectangles in HPKs indicate predicted or experimentally determined Trans Membrane Segments (see also Table 3.1). Below each HPK and RR the genomic organization of the HPK and RR and their flanking regions are shown. The description of relevant genes can be found in the text. Stemloops indicate potential transcription termination sites.

Results and discussion

TCSs characteristic of peptide-based quorum sensing

Four genetically linked, complete TCSs (*pln*, *plt*, TCS10 and *lam*) were predicted to encode peptide-based quorum sensing systems, based on the presence of a HPK_{10}-subfamily type HPK adjacent to a RR containing a LytTR DNA-binding domain. For three of these (*pln*, *plt* and *lam*), an upstream gene encoding a putative autoinducing peptide could be identified (Figs. 3.3 and 3.4). For two TCSs (*pln* and *plt*), the AIP sequences contained a putative double-glycine-type leader-peptide (43), while the third TCS (*lam*) contained a preceding gene that encoded for a putative cyclic thiolactone AIP (27). In addition, one orphan HPK with HPK_{10}-subfamily characteristics (*hpk9*) and one orphan RR containing a HTH-LytTR DNA-binding domain (*rrp8*) were detected. Relevant features of these genes that address their functionality are discussed here.

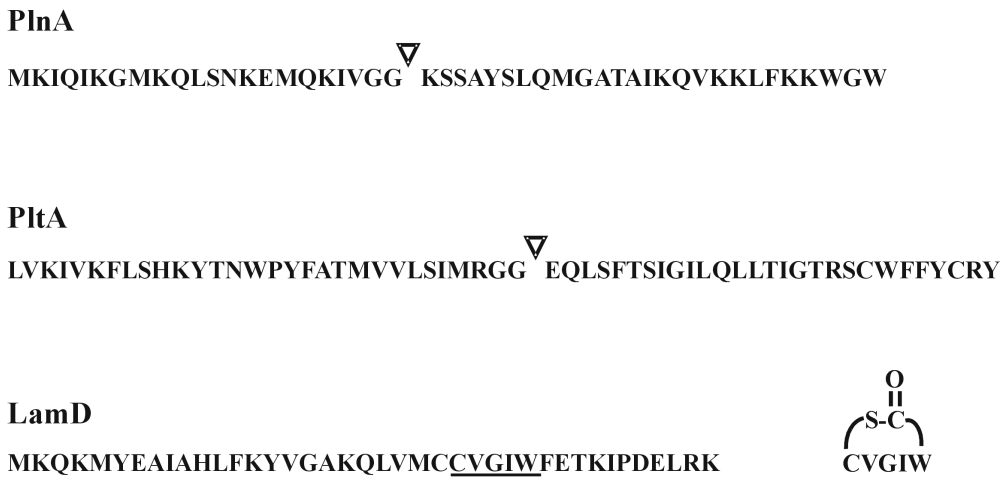


Figure 3.4 Autoinducing peptides encoded on the *L. plantarum* WCFS1 genome. Triangles indicate the cleavage site between the double-glycine leader-peptide and mature peptide in PlnA and PltA. The underlined residues in the LamD precursor peptide are processed to the mature peptide shown on the right.

Plantaricin TCS pln (lp_0415 to lp_0418)

The plantaricin TCS *pln* (lp_0415 to lp_0418) was identical to the previously described *plnABCD* regulatory system of *L. plantarum* C11 that regulates the production of class II antimicrobial peptides (AMP) (11). This system contained a gene coding for a HPK (*plnB*: lp_0416) that showed the HPK_{10}-subfamily characteristics and 7 TMS (experimental confirmation). Downstream of *plnB*, two RRs instead of one were encoded (*plnC*: lp_0417 and *plnD*: lp_0418). Each contained a CheY-like receiver domain and a LytTR HTH-DNA-binding domain. Upstream of *plnB*, an AIP precursor peptide of 48 amino acids was encoded (*plnA:* lp_0415), which contained a double-glycine-type leader peptide as is found for AIPs involved in class II AMP production (43). PlnA is however not one of the main plantaricin bacteriocins. Genes encoding these plantaricins (PlnE-PlnF, PlnJ-PlnK, and PlnN) are localized outside the regulatory module (Fig. 3.3A). Cleavage of the double-glycine-type leader-peptide from the PlnA precursor peptide results in a linear AIP of 26 amino acids without modifications (Fig. 3.4). The complete *pln* regulatory module was >99.5% identical on the nucleotide-level to the *pln*-system from strain C11 and consists of 24 genes that are arranged in 5 sequential clusters with different orientations: *plnRLKJ*, *plnMNOP-orf1*, *plnABCD*, *plnIFE* and *plnGHSTUVWXY*. The most remarkable differences are that, compared to strain C11, the WCFS1 *pln*-cluster has a complete *plnV* gene, three additional genes *plnWXY*, and a *plnL* gene that is 90 residues longer (33). The *pln*-system was found to be functional in *L. plantarum* WCFS1 as was shown by a bacteriocin-diffusion assay with *Lactobacillus plantarum* 965 as an indicator strain (Fig. 3.5). The *L. plantarum* WCFS1 native state appeared to be bacteriocin-negative (Bac^-), but bacteriocin production could be induced with either bacteriocin-positive supernatant of strain C11 (Fig. 3.5F) or purified PlnA peptide (Fig. 3.5C). Halos produced by strain WCFS1 appeared to be smaller than those seen for C11, but nevertheless, inducible bacteriocin production under control of PlnA was shown. Comparative analysis of 20 different *L. plantarum* strains showed that the *pln* locus is absent or incomplete in many of them (41). The *pln*-system is expected to have a role in competition with other bacteria, as it regulates the production of the class II AMPs PlnE-PlnF, PlnJ-PlnK, and PlnN. These plantaricins showed strain-specific activity against the closely related *L. plantarum* 965, *L. sake* NCDO 2714 and *Pediococcus pentosaceus* strains Pac 1.0 and NCDO 990 (4), which

can be found in the same ecological niches in the GI-tract as *L. plantarum* strains WCFS1 and C11 (63).

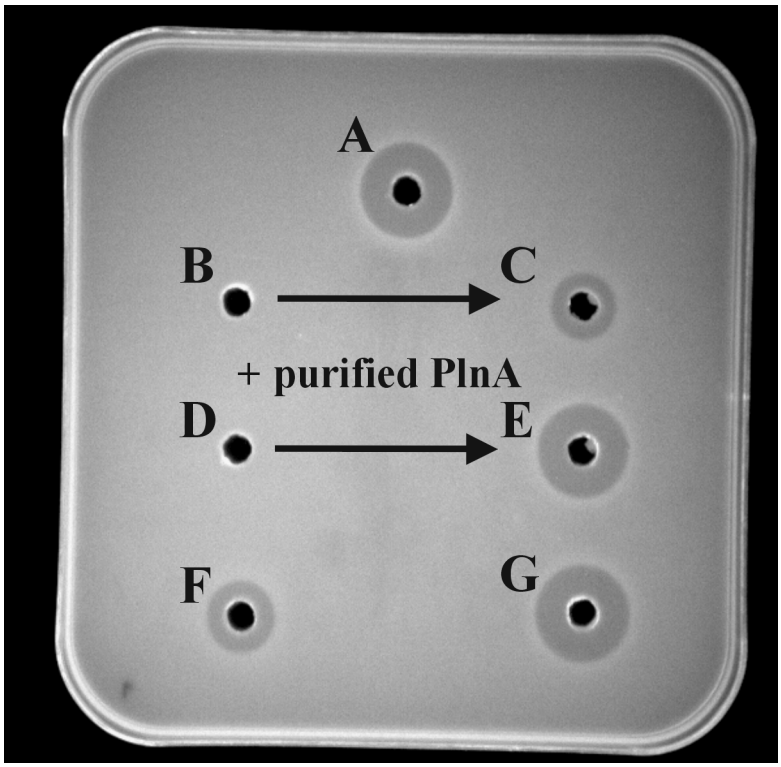


Figure 3.5 Assay for plantaricin-production in *L. plantarum* WCFS1 and the related strain C11. A) C11, Bac^+; B) WCFS1, Bac^-; native state of WCFS1; C) Pln production in WCFS1, induced with purified PlnA peptide ; D) C11, Bac^-; obtained by $<10^4$ cfu/ml dilution; E) Pln production in C11 ($<10^4$ cfu/ml dilution), induced with purified PlnA; F) Pln production in WCFS1, induced with C11, Bac^+ supernatant; G) Pln production in C11, induced with WCFS1, Bac+ supernatant. Strain C11, target strain 965 and purified PlnA were generous gifts from Dzung Diep and Ingolf Nes.

TCS plt (lp_1354a, lp_1355 and lp_1356)

TCS *plt* (lp_1354a, lp_1355 and lp_1356) contained genes encoding a HPK (*pltK:* lp_1355) that showed the HPK_{10}-subfamily characteristics and 5-6 predicted TMS, and a RR (*pltR*: lp_1356) that contained a CheY-like receiver domain and a LytTR HTH-DNA-binding domain. Upstream of *pltK*, an AIP precursor peptide of 58 amino acids was encoded (*pltA*: lp_1354a), which contained a double-glycine-type leader peptide as is found for AIPs involved in class II AMP production (43). Cleavage of the double-glycine-type leader-peptide from the precursor peptide is

predicted to result in a linear AIP of 28 amino acids without modifications (Fig. 3.4). Northern hybridizations showed that *pltA*, *pltK* and *pltR* are co-transcribed as a single large transcript of 2.4 kb that displayed cell-density dependent expression (Fig. 3.6). The predicted linear AIP was chemically synthesized, but appeared to be water-insoluble and act as a gel above 35-40% purity, and therefore could not be used in induction experiments. This could be a consequence of the high number of hydrophobic residues in this peptide and the lack of a clear amphipatic character when projected as an alpha helix, which is a common characteristic of the class II bacteriocin-like AIPs, like PlnA (Fig. 3.7) (4, 66). Alternatively, the *pltA*-encoded peptide might undergo unpredicted post-translational modifications that change its solubility characteristics and might render a functional secreted AIP. In conclusion, it remains unclear whether the predicted mature PltA peptide or a modified form of this peptide plays a role in regulation of the cell-density dependent expression of the *pltAKR* operon. However, based on the genetic organization and its analogy with other systems it is tempting to speculate that the *pltAKR* operon encompasses a functional QS-TCS.

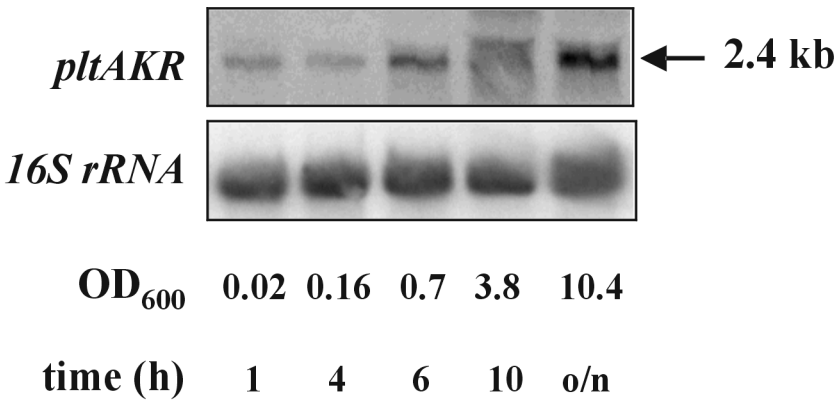


Figure 3.6 Northern blot analysis of temporal expression of the *plt*-cluster. Expression of *pltAKR* during growth using a *pltK* internal probe is shown. The same 2.4-kb fragment was found to hybridize with either the *pltA* or *pltR* probes (data not shown).

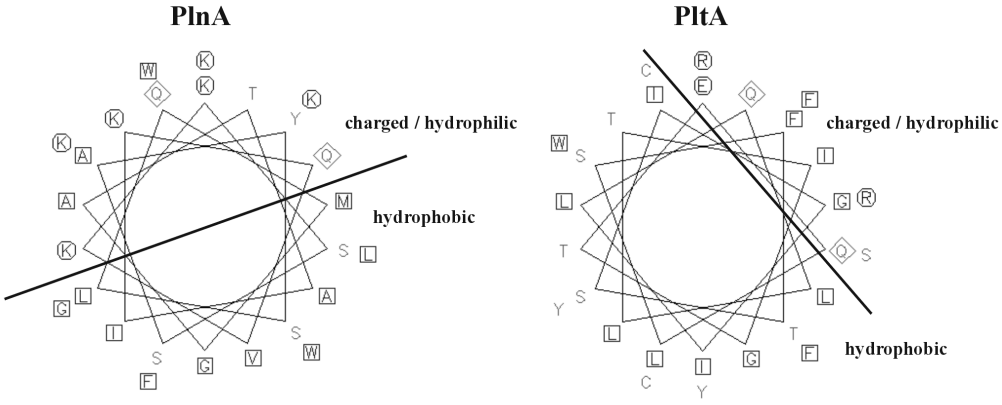


Figure 3.7 Alpha-helical wheel-representation of the (predicted) mature linear autoinducing peptides PlnA and PltA. The wheel was plotted using the program PepWheel (EMBOSS) at http://bioweb.pasteur.fr/seqanal/interfaces/pepwheel.html. Squares: hydrophobic residues; diamonds: hydrophilic residues; octagons: charged residues. The line indicates the amphipathic division between the charged / hydrophilic side and the hydrophobic side of the alpha-helix.

The *plt*-system displays the characteristics of TCS involved in class II AMP production, but there is no indication for its regulatory role. For *plt* we did not find an associated ABC-transporter for AIP or bacteriocin transport, as is common for these systems (43). It might be that another ABC-transporter with a Peptidase C39 protease-family domain (22) could be used for this purpose, such as the transporter for the *pln*-system (encoded by *plnG* and *plnH* in Fig. 3.3A). At this moment it therefore stays unclear whether *plt* could play a role in competition with other bacteria.

TCS lam (lp_3580 to lp_3582)

The TCS encoded by lp_3580 to lp_3582 was named *lam* for *Lactobacillus agr*-like module, as it showed a similar gene organization as the *agrBDCA* quorum sensing system of *Staphylococcus aureus* (27), but also other recognized *agr*-like quorum sensing systems such as *fsrABC* of *Enterococcus faecalis* (53) and *comQXPA* of *Bacillus subtilis* (5). This TCS contains genes coding for a HPK (*lamC*: lp_3581) that showed the HPK_{10}-subfamily characteristics and 5 predicted TMS, and a RR (*lamA*: lp_3580) that contained a CheY-like receiver domain and a LytTR HTH-DNA-binding domain. These genes were shown to belong to an operon with additional upstream-located genes encoding an AgrD-like precursor peptide (*lamD*:

lp_3581a) and an *agrB*-orthologue (*lamB*: lp_3582) (59). Experimental data showed that a cyclic thiolactone peptide with sequence CVGIW was produced by *L. plantarum* WCFS1, which in analogy with the staphylococcal *agr*-system (27) is likely to result from processing of the LamD precursor peptide by LamB (Fig. 3.4). Expression of the *lamBDCA* genes and production of the *lamD*-encoded peptide were shown to be cell-density dependent, with a clear increase from mid-log to late-log growth phase. Analysis of a response regulator-defective mutant (Δ*lamA*) in an adherence-assay showed that *lam* regulates the capacity of *L. plantarum* to adhere to surfaces. Global transcription analysis of a wild-type and *lamA*-mutant strain in early-, mid- and late-log phase of growth using DNA microarrays showed that only a small set of clustered genes (2% of all genes) showed significant differences in transcription profiles between the wild-type and *lamA*-mutant. The microarray analysis confirmed that *lamBDCA* is autoregulatory (59). The finding of a direct repeat sequence within the promoter region of the *lam* operon that displays similarity with previously established regulatory target sites of LamA homologues (42, 53-55) suggests that this direct repeat acts as the LamA regulatory DNA-target site (Fig. 3.8). In addition to regulation of its own expression, *lamA* appeared to be involved in regulation of expression of genes encoding surface polysaccharides, cell membrane proteins and sugar-utilization proteins. This is the first example of an *agr*-like system in lactobacilli, which encodes a cyclic thiolactone autoinducing peptide and is involved in regulation of adherence properties (59).

TCS10 (lp_3087 and lp_3088)

Like the *lamCA*-encoded proteins, TCS10 (lp_3087 and lp_3088) displays homology with the *S. aureus agr*-system, but lacked the *agrBD* gene-homologs that were present in the *lamBDCA* locus. HPK10 (*hpk10*; lp_3088) displays HPK_{10}-subfamily characteristics and 6 predicted TMS, and RRP10 (*rrp10*; lp_3087) contained a CheY-like receiver domain and a LytTR HTH-DNA-binding domain. TCS10 and *lamBDCA* could represent (partial) paralogous systems, as HPK10 and RRP10 showed 55% and 70% overall identity on the amino acid level to LamC and LamA, respectively. In addition, the promoter-region upstream of the *hpk10* gene contains a direct repeat that displays high similarity to the repeat found upstream of the *lamBDCA* locus (Fig. 3.8). Considering the high similarity between the HTH-

LytTR DNA-binding domains of LamA and RRP10 (Fig. 3.9B), combined with the presence of similar direct repeat sequences within the putative promoters of these genetic loci, one could imagine that these RRs recognize the same or highly similar direct repeats and might be involved in regulation of a similar (or identical) set of target genes. However, DNA microarray analyses of wild-type versus *lamA*-mutant did not show significant differences in gene expression of the TCS10-encoding locus in the *lamA*-mutant as compared to the wild-type strain, which indicated that there is most probably no regulon overlap between TCS10 and *lamBDCA*.

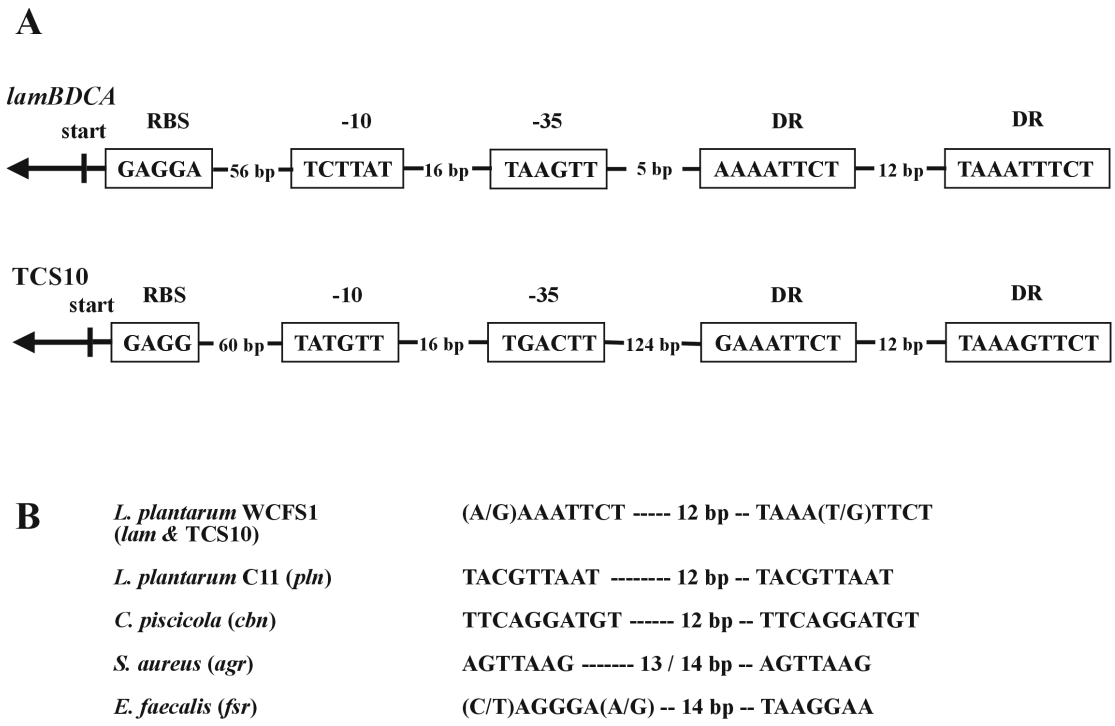
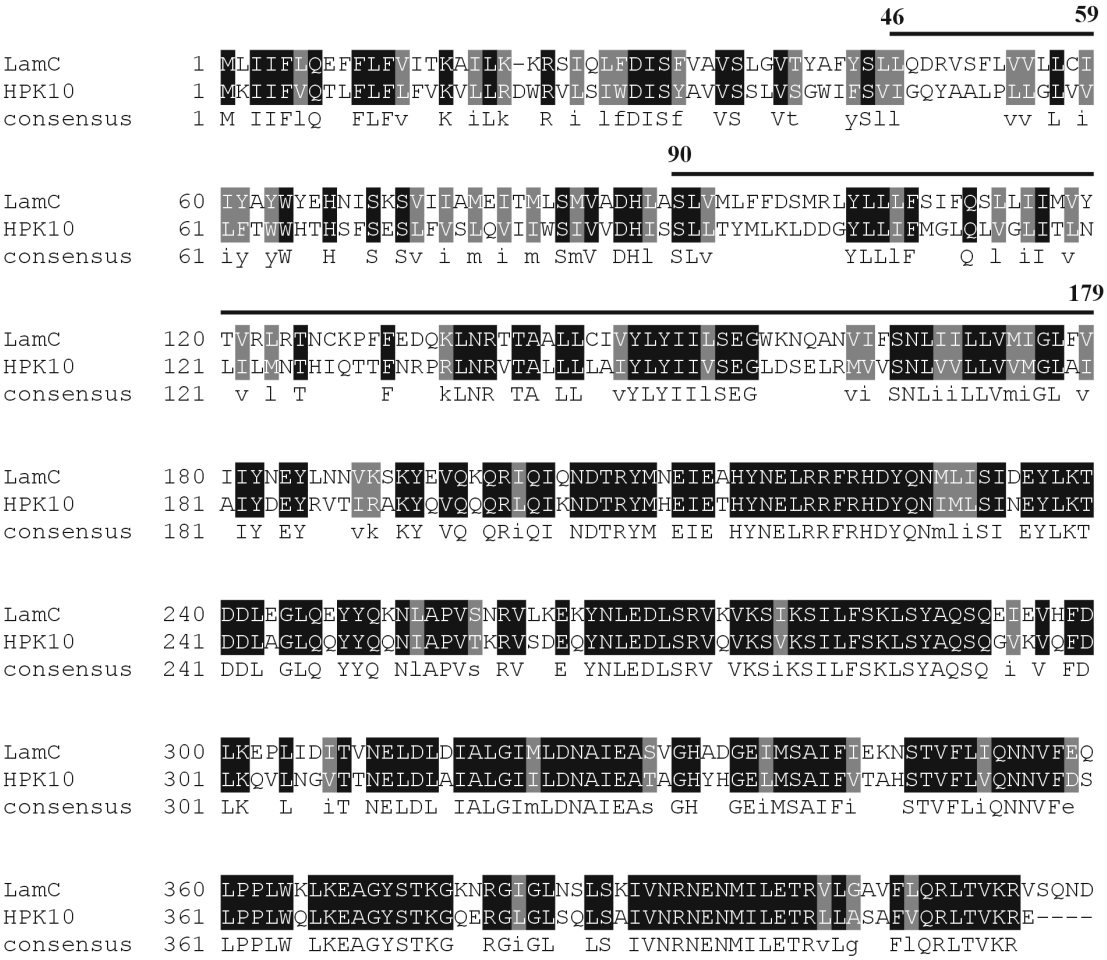


Figure 3.8 (A) Direct repeats (DR) detected in promoter regions of *lamBDCA* and TCS10. RBS: Ribosome Binding Site. TATA-boxes are indicated by –10 and –35. The spacing between different elements is indicated in bp. (B) Direct repeats in promoters of *agr*-like quorum sensing TCS.

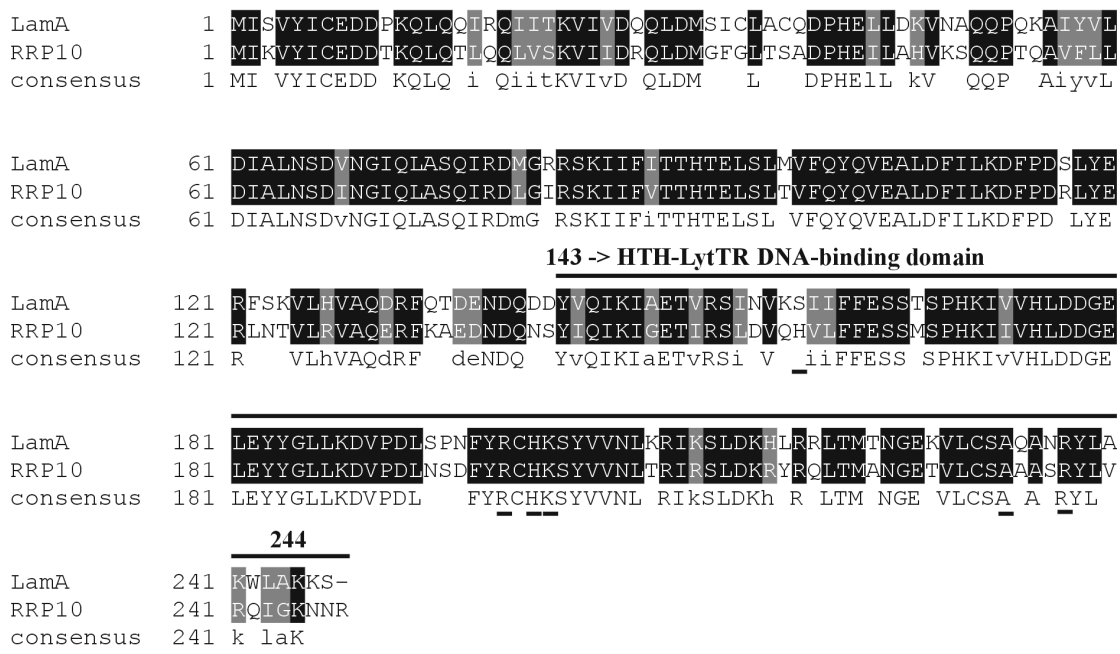
For AgrC-like HPKs no clear data exist on residues that are essential for AIP-binding or specificity. The only data available show that specificity in AgrC resides in the distal region of the N-terminal receptor-domain, which would correspond to approximately residues 90-180 in both *lamC* and *hpk10* (70). In contrast, for the related PlnB of *L. plantarum* and ComD of *S. pneumoniae*, data indicated that more proximal regions would be responsible for AIP-binding and specificity (29). The highest homology between LamC and HPK10 is found in their transmitter domain rather than their receptor domain (Fig. 3.9A). Neither of the postulated receptor-ligand interaction domains (proximal or distal; Fig. 3.9A) of LamC and HPK10 displays very high overall identity, suggesting that these HPK respond to different input signals and that HPK10 would thus not interact with the cyclic peptide identified as the *lamBDCA* derived signaling molecule.

Figure 3.9 (*opposite page*) Protein sequence alignments of (A) LamC and HPK10 and (B) LamA and RRP10. Alignments and shading were performed using ClustalW (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) and BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Highly conserved residues are shown in *black* (identical) or *grey* (similar physico-chemical characteristics).

A


46
59
90
179
LamC 1 MLIIFLQEFFLFVITKAILK-KRSIQLFDISFVAVSLGVTYAFYSLLQDRVSFLVVLLCI
HPK10 1 MKIIFVQTLFLFLFVKVLLRDWRVLSIWDISYAVVSSLVSGWIFSVIGQYAALPLLGLVV
consensus 1 M IIFlQ FLFv K iLk R i lfDISf VS Vt ySll vv L i
LamC 60 IYAYWYEHNISKSVILAMEITMLSMVADHLASLVMLFFDSMRLYLLLFSIFQSLLIIMVY
HPK10 61 LFTWWHTHSFSESLFVSLQVIIWSIVVDHISSLLTYMLKLDDGYLLIFMGLQLVGLITLN
consensus 61 iy yW H S Sv i m i m SmV DHl SLv YLLlF Q l iI v
LamC 120 TVRLRTNCKPFFEDQKLNRTTAALLCIVYLYIILSEGWKNQANVIFSNLIILLVMIGLFV
HPK10 121 LILMNTHIQTTFNRPRLNRVTALLLLAIYLYIIVSEGLDSELRMVVSNLVVLLVVMGLAI
consensus 121 v l T F kLNR TA LL vYLYIIlSEG vi SNLiiLLVmiGL v
LamC 180 IIYNEYLNNVKSKYEVQKQRIQIQNDTRYMNEIEAHYNELRRFRHDYQNMLISIDEYLKT
HPK10 181 AIYDEYRVTIRAKYQVQQQRLQIKNDTRYMHEIETHYNELRRFRHDYQNIMLSINEYLKT
consensus 181 IY EY vk KY VQ QRiQI NDTRYM EIE HYNELRRFRHDYQNmliSI EYLKT
LamC 240 DDLEGLQEYYQKNLAPVSNRVLKEKYNLEDLSRVKVKSIKSILFSKLSYAQSQEIEVHFD
HPK10 241 DDLAGLQQYYQQNIAPVTKRVSDEQYNLEDLSRVQVKSVKSILFSKLSYAQSQGVKVQFD
consensus 241 DDL GLQ YYQ NlAPVs RV E YNLEDLSRV VKSiKSILFSKLSYAQSQ i V FD
LamC 300 LKEPLIDITVNELDLDIALGIMLDNAIEASVGHADGEIMSAIFIEKNSTVFLIQNNVFEQ
HPK10 301 LKQVLNGVTTNELDLAIALGILLDNAIEATAGHYHGEIMSAIFVTAHSTVFLVQNNVFDS
consensus 301 LK L iT NELDL IALGImLDNAIEAs GH GEiMSAIFi STVFLiQNNVFe
LamC 360 LPPLWKLKEAGYSTKGKNRGIGLNSLSKIVNRNENMILETRVLCAVFLQRLTVKRVSQND
HPK10 361 LPPLWQLKEAGYSTKGQERGLGLSQLSAIVNRNENMILETRLLASAFVQRLTVKRE----
consensus 361 LPPLW LKEAGYSTKG RGiGL LS IVNRNENMILETRvLg FlQRLTVKR

B


LamA 1 MISVYICEDDPKQLQQIRQIITKVIVDQQLDMSICLACQDPHELLDKVNAQQPQKAIYVL
RRP10 1 MIKVYICEDDTKQLQTLQQLVSKVIIDRQLDMGFGLTSADPHEILAHVKSQQPTQAVFLL
consensus 1 MI VYICEDD KQLQ i QiitKVIvD QLDM L DPHElL kV QQP AiyvL
LamA 61 DIALNSDVNGIQLASQIRDMGRRSKIIFITTHTELSLMVFQYQVEALDFILKDFPDSLYE
RRP10 61 DIALNSDINGIQLASQIRDLGIRSKIIFVTTHTELSLTVFQYQVEALDFILKDFPDRLYE
consensus 61 DIALNSDvNGIQLASQIRDmG RSKIIFiTTHTELSL VFQYQVEALDFILKDFPD LYE
143 -> HTH-LytTR DNA-binding domain
LamA 121 RFSKVLHVAQDRFQTDENDQDDYVQIKIAETVRSINVKSIIFFESSTSPHKIVVHLDDGE
RRP10 121 RLNTVLRVAQERFKAEDNDQNSYIQIKIGETIRSLDVQHVLFFESSMSPHKIIVHLDDGE
consensus 121 R VLhVAQdRF deNDQ YvQIKIaETvRSi V iiFFESS SPHKIvVHLDDGE
LamA 181 LEYYGLLKDVPDLSPNFYRCHKSYVVNLKRIKSLDKHLRRLTMTNGEKVLCSAQANRYLA
RRP10 181 LEYYGLLKDVPDLNSDFYRCHKSYVVNLTRIRSLDKRYRQLTMANGETVLCSAAASRYLV
consensus 181 LEYYGLLKDVPDL FYRCHKSYVVNL RIkSLDKh R LTM NGE VLCSA A RYL
244
LamA 241 KWLAKKS-
RRP10 241 RQIGKNNR
consensus 241 k laK

Orphan genes rrp8 (lp_2665) and hpk9 (lp_3063)

In addition to the above-mentioned genetically linked TCSs, two orphan genes were identified that showed characteristics of peptide-based quorum sensing systems.

The orphan gene *hpk9* (lp_3063) was predicted to encode a HPK with HPK_{10}-subfamily characteristics and 6 TMS (Fig 3.3A). Interestingly, immediately downstream (7 bp) of *hpk9*, gene lp_3062 is located, which encodes a small ORF of 94 residues that shows 46% homology to the C-terminal domain of the TfoX-protein of *Haemophilus influenzae* (Pfam accession number PF04994). The TfoX-protein has been suggested to play a key role in cell density-dependent regulation of genetic competence in *H. influenzae*, and the C-terminal domain is suggested to function autonomously (30, 72). The lp_3062 gene-product might therefore function in co-operation with *hpk9*. However, no clearly identifiable cognate RR appears to be encoded in the vicinity of *hpk9*. The downstream-located response regulator lp_3060 probably does not serve this function, as it was not transcriptionally linked (data not shown) and does not contain a clear CheY-like receiver domain or quorum sensing-related LytTR or HTH-LuxR DNA-binding domain. In contrast, this regulator contains a typical HTH-AraC DNA-binding domain (COG2207) (Fig. 3.3). It might be that the *hpk9*-encoded HPK forms a functional TCS with the *rrp8*-encoded protein, and could be involved in peptide-based quorum sensing, with the genes being separated on the chromosome during evolution. Notably, the orphan gene *rrp8* (lp_2665) was predicted to encode a RR with a typical LytTR HTH-DNA-binding domain. A Neighbour Joining phylogenetic tree (NJ-tree) based on protein sequence alignments of (quorum sensing) TCS from over 200 deposited bacterial genomes, showed that the *hpk9*- and *rrp8*-encoded proteins clustered with a HPK and RR of *Enterococcus faecium* that are adjacent on the *E. faecium* chromosome (data not shown), which would support the hypothesis that *hpk9* and *rrp8* actually form a functional TCS. Interestingly, the gene immediately downstream of *rrp8* (lp_2664, with only 13 bp intergenic region to lp_2665) contains a HDc-type phosphohydrolase-domain (SMART accession number SM00471) that might have a role in dephosphorylation of the *rrp8* gene-product. Overall, the role of *hpk9* in QS-mediated regulation and the identity of its eventual partnering transcriptional regulator remain to be established.

Concluding remarks

The relatively high number of predicted peptide-based QS-TCS in *L. plantarum* (5, of which at least 3 contain a predicted AIP) might be related to the ecological flexibility displayed by this micro-organism that can be found in plants, fermented foods and the gastrointestinal tract. Furthermore, depending on the environmental conditions different signaling systems might be triggered, resulting in diverse cell-density dependent regulated phenotypic traits. An example of such variation might be the involvement of the *lamBDCA*-system in adherence of *L. plantarum* to surfaces under specific conditions (59), while the presence of competing micro-organisms could activate specific QS-TCS involved in competition, as has been shown for the plantaricin-system in *L. plantarum* NC8 (38).

Besides these peptide-based QS-TCSs, TCS4 (lp_1487 and lp_1488) and TCS6 (lp_1942 and lp_1943) were detected that both encoded for a HPK_7-subfamily type HPK (20) adjacent to a RR containing a HTH-LuxR DNA-binding domain (17) (see Table 3.1 and Fig. 3.3B). This resembled the architecture of the ComPA QS-TCS of *B. subtilis* (67, 68). Moreover, the HPK encoded by *hpk6* (lp_1943) was also predicted to contain 4-5 TMS, which is similar to ComP that has 8-10 TMS (51). For HPKs belonging to the HPK_7-subfamily, the available literature data suggest that they do not seem to be part of QS-TCS (20, 69), with the only exception to date being ComP in *B. subtilis*. The HTH-LuxR DNA-binding domain has been detected in the peptide-based quorum sensing RR ComA in *B. subtilis* and transcriptional regulators that respond to *N*-acyl homoserine lactones as a quorum sensing signal (19) in Gram-negative bacteria. However, it has also been found in non-quorum sensing transcriptional regulators and RRs (6, 12). Therefore, the *in silico* analysis of TCS4 and TCS6 at present does not reveal their involvement, or lack of it, in quorum sensing regulation of gene expression in *L. plantarum*.

Interestingly, next to the peptide-based quorum sensing TCS, also an isolated homologue of the *luxS* gene was identified (lp_0774), encoding the autoinducer-2 (AI-2) synthase (56). However, associated functions like *lsr*, which is involved in AI-2 uptake in *Salmonella typhimurium* (61), or *luxPQ* involved in AI-2 sensing and signal transduction in *Vibrio harveyi* (7) were not identified in the *L. plantarum* genome (55). Transcription of *luxS* was confirmed by Northern hybridization, showing that the *luxS* gene was monocistronically expressed.

Moreover, an AI-2 reporter-assay in *Vibrio harveyi* (7) showed that *L. plantarum* WCFS1 produces an AI-2-like molecule, whereas this production was abolished in a *luxS* knockout-mutant (data not shown). Autoinducer-2 could be involved in interspecies communication of *L. plantarum* with other species within the same niche, as the *luxS* gene has been found in both Gram-negative and Gram-positive bacteria (71) and was shown to play a role in for example formation of mixed biofilms of *Streptococcus gordonii* and *Porphyromonas gingivalis* (39). In addition, AI-2 activity was detected in rumen samples (40), suggesting a natural role of the AI-2 quorum sensing system in gastrointestinal ecosystems.

Further experimental studies on the regulatory mechanisms of the different quorum sensing systems of *L. plantarum* and their effects on (global) gene regulation will be necessary, to provide more insight into the role of these systems in the survival of this organism in its natural environments.

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An *agr*-like two-component regulatory system in *Lactobacillus plantarum* is involved in production of a novel cyclic peptide and regulation of adherence

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Abstract

We have analyzed a locus on the annotated *Lactobacillus plantarum* WCFS1 genome that showed homology to the staphylococcal *agr* quorum sensing system, and designated it *lam* for *Lactobacillus agr*-like module. Production of the *lamBDCA* transcript was shown to be growth-phase dependent. Analysis of a response regulator-defective mutant (Δ*lamA*) in an adherence-assay showed that *lam* regulates adherence of *L. plantarum* to a glass-surface. Global transcription analysis of wild-type and Δ*lamA* strain in early-, mid- and late-log phase of growth was performed using a clone-based microarray. Remarkably, only a small set of genes showed significant differences in transcription profiles between the wild-type and *lamA* mutant. The microarray analysis confirmed that *lamBDCA* is autoregulatory and showed that *lamA* is involved in regulation of expression of genes encoding surface polysaccharides, cell membrane proteins and sugar-utilization proteins. The *lamBD* genes encoding the putative autoinducing peptide precursor (LamD) and its processing protein (LamB) were overexpressed using the Nisin Controlled Expression system and culture supernatants analyzed by LC/MS to identify overproduced LamD-derived peptides. In this way a cyclic thiolactone pentapeptide was identified, which possesses a ring structure similar to autoinducing peptides of the staphylococcal *agr*-system. The peptide was designated LamD558 and its sequence (CVGIW) matched the annotated precursor peptide sequence. Time course analysis of wild-type culture supernatants by LC/MS indicated that LamD558 production was increased markedly from mid-log to late-log growth-phase. This is the first example of an *agr*-like system in non-pathogenic bacteria, which encodes a cyclic thiolactone autoinducing peptide and is involved in regulation of adherence.

Introduction

Regulation of physiological changes in bacterial populations in many cases has been shown to be dependent on specific cell densities and growth phases. This phenomenon of cell density-dependent gene expression has been termed quorum sensing, and was initially found to regulate bioluminescence in *Vibrio fischeri* (15). Since then, a large variety of quorum sensing systems has been discovered in both Gram-negative and Gram-positive bacteria (29). Well-studied examples of quorum sensing regulated features in Gram-positive bacteria include genetic competence in *Bacillus subtilis* (55) and *Streptococcus pneumoniae* (8), virulence and biofilm formation in *Staphylococcus aureus* (39, 65) and *Enterococcus faecalis* (16, 44), and the production of antimicrobial peptides, including bacteriocins and lantibiotics, in various lactic acid bacteria (26, 38). To regulate these quorum sensing systems bacteria produce extracellular signaling molecules that are responsible for cell-to-cell communication. While many Gram-negative bacteria communicate via *N*-acyl-homoserine lactones, peptides are the most common and well-studied signaling molecules in Gram-positive bacteria, here referred to as autoinducing peptides (AIPs). These peptides have diverse structures but share a small size, ribosomal synthesis, and -in many cases- are subject to specific post-translational modifications that add to their stability, specificity and functionality. The exported AIPs regulate quorum sensing via two-component regulatory systems (TCS), consisting of histidine protein kinase (HPK) and response regulator (RR) genes (26, 54).

Lactobacilli are commonly used in fermentations of dairy, meat and vegetable foods (7, 47), but several species are also common inhabitants of the human gastrointestinal tract (58). One of these is *Lactobacillus plantarum* (1), which is a versatile species that is encountered in diverse environmental niches like fermented food products, on plant material, and in the human gastrointestinal tract (1, 13, 14). *L. plantarum* WCFS1 is a single colony isolate of the esophageal *L. plantarum* strain NCIMB8826 (17), which was shown to survive stomach passage in an active form and to persist for up to 7 days in the human GI-tract after single dosage (6, 59). The *L. plantarum* WCFS1 genome sequence has been completed and appears to be one of the largest genomes known among lactic acid bacteria (27). The 3.3-Mb genome encodes a large repertoire of extracellular proteins, sugar and amino acid import and utilization proteins, and many regulatory genes, including 13 TCS. These may aid to

the persistence and survival of this highly adaptive microbe in diverse ecological niches (27).

Here we describe the functional analysis of an annotated two-component regulatory system of *L. plantarum* WCFS1 (27) that shows homology to the *agrBDCA* and *fsrABC* quorum sensing systems of *Staphylococcus aureus* (21) and *Enterococcus faecalis* (34), respectively (Fig. 4.1). The latter quorum sensing systems are involved in regulation of virulence factor production, mainly via the production of extracellular proteins (40, 44). In addition, they are also involved in biofilm formation (16, 60, 65). The *agr*-system in staphylococci encodes the two-component histidine protein kinase AgrC and response regulator AgrA, an AIP derived from precursor peptide AgrD, and additionally AgrB, a protein that is involved in processing and post-translational modification of AgrD (66, 67). The corresponding regulatory proteins and a fused FsrBD protein can also be found in the *fsr*-system (34, 45). The AIPs produced from the precursor peptide AgrD are cyclic thiolactone peptides in staphylococci (20), while in *E. faecalis* the C-terminal part of FsrBD encompasses the cyclic lactone gelatinase biosynthesis-activating pheromone (34). One of the *L. plantarum* TCS showed a similar genetic organization as the *agr*-system and was designated *lam* for *Lactobacillus agr*-like module. The function and expression of the *lam*-system was studied by a global genomics approach and *lam* was found to be involved in regulation of adherence to a glass-surface. Moreover, the production of a novel *agr*-like cyclic thiolactone pentapeptide could be established by homologous over-expression of the *lamBD* genes.

Materials and Methods

Bacterial strains and growth media

The bacterial strains and plasmids used in this study and their characteristics are shown in Table 4.1. *Escherichia coli* JM109 was cultivated at 37°C aerobically in Luria-Bertani broth (LB) or in Brain-Heart-Infusion broth when selecting for erythromycin-resistance (BHI; Difco). *Lactobacillus plantarum* WCFS1 and its derivatives were cultivated in Man-Rogosa-Sharpe broth (MRS; Difco) at 30°C without agitation (unless stated differently). Solid media contained 1.5% (w/v) agar. Where appropriate, antibiotics were added as follows: erythromycin 150 μg ml^{-1} (*E.*

coli) or 5 μg ml^{-1} (*L. plantarum* WCFS1); chloramphenicol 25 μg ml^{-1} (*E. coli*) or 10 μg ml^{-1} (*L. plantarum* WCFS1).

DNA isolation and construction of integration plasmids

Genomic DNA was isolated from 10 ml logarithmic phase cultures of *L. plantarum* WCFS1 essentially as described (4), with additional disruption by bead-beating in a Biospec bead-beater (3x 90 sec at 5000 rpm) and purification by phenol-chloroform extractions and isopropanol precipitation. PCR was performed using proofreading Platinum *Pfx* DNA Polymerase (Invitrogen) and as a template genomic DNA or plasmid pBACe3.6 (generously provided by Dr. J. Catanese). Primer combinations WCFS-lamA-*Nde*I with WCFS-lamA-*Eco*RI and WCFS-lamA-*Kpn*I with WCFS-lamA-*Bam*HI were used to amplify the 5'- and 3'-ends of *lamA* and the regions flanking *lamA* (approximately 1 kb on each side), whereas primer-combination pBAC-SacB-*Eco*RI with pBAC-SacB-*Kpn*I was used to amplify a locus-tagging-sequence that was cloned in between these two fragments (Table 4.2). PCR products were cloned into the non-replicating integration-vector pUC19ery, after digesting the PCR products and vector with the appropriate restriction enzymes (Gibco BRL) (56). Plasmids were transformed into *E. coli* JM109 by a heatshock procedure as recommended by the manufacturer (Promega). This resulted in plasmid pLAMA-K28 containing the complete gene replacement cassette with the mutated response regulator gene.

Gene replacement of the *L. plantarum* WCFS1 *lamA* gene

L. plantarum WCFS1 was transformed by electroporation, essentially as previously described (22), using electro-competent cells of *L. plantarum* that were grown in MRS supplemented with 1% glycine and prepared in 30% polyethyleneglycol-1450 (PEG-1450). *L. plantarum* cells were transformed with 2 μg of integration plasmid pLAMA-K28, and integrants were selected by plating on MRS-agar supplemented with erythromycin and incubation in an anaerobic jar at 30°C for 2-4 days. Single colonies were analyzed by colony PCR with plasmid-chromosome crossover-junction primers Lam-*Nde*I-junction-1 and SacB-control-2 or SacB-control-3 and Lam-*Bam*HI-junction-4 (Table 4.2) to confirm single crossover integration up- or downstream of the mutation locus. For this purpose, single colonies were suspended in 20 μl TE and cells disrupted by a microwave-treatment for 3 min at 750

W and subsequent incubation for 10 min at 95°C, after which PCR analysis was performed. A single crossover mutant was selected and designated *L. plantarum* WCFS1::pLAMA-K28. This mutant was subsequently propagated for 200 generations in MRS without erythromycin to obtain the anticipated erythromycin-sensitive (Em^S) *lamA*::tag replacement mutant after a second homologous recombination event. The anticipated gene organization of the *lamA*::tag locus in the obtained gene replacement mutant (MSΔlamA) was verified by colony PCR with crossover-junction primers and *lamA*-flanking primers RR-lamA-F and RR-lamA-R as described before (primers shown in Table 4.2), as well as by Southern blot analysis using standard procedures (50).

RNA extraction

Cultures of the wild type and *lamA*-mutant strains were aerobically grown in 100 ml MRS at 30ºC starting from an OD_{600} of 0.3. Samples (25 ml) were harvested at early-, mid- and late-logarithmic growth-phases and immediately quenched and mixed in 4 volumes of quenching-buffer at –80ºC to stop further transcription (60% methanol, 66.7 mM HEPES pH 6.5), as described recently (42). Subsequently samples were pelleted by centrifugation at -20ºC in a pre-chilled centrifuge and cells resuspended in 0.5 ml cold TE-buffer. RNA was isolated according to the Macaloid-method, essentially as described (28), and further purified by on-column DNaseI treatment on RNeasy columns (Qiagen). For DNA microarray analysis 25 μg RNA aliquots were prepared. All experiments were performed in duplicate.

Northern blot analysis of *lamBDCA* gene expression

RNA was isolated as described above and 10.0 μg RNA sample or 3.0 μl RNA size-marker (Invitrogen) were used for Northern blot analysis by glyoxal denaturation as described (50). RNA was fixed to the membrane by auto-crosslinking in an UV Stratalinker and Northern hybridization performed overnight at 65°C in QuikHyb hybridization buffer (Stratagene) as described (50). Blots were analyzed by exposure to Phosphor screens (Molecular Dynamics) and scanned on a Storm Image Scanner (Molecular Dynamics). DNA probes were made using a Nick Translation kit (Invitrogen). Signal intensities were quantified for a *lamC* internal probe and normalized against the 16S rRNA signal intensities.

Table 4.1. Bacterial strains and plasmids used in this study and their relevant characteristics

Bacterial strains & plasmids	Relevant properties [a]	Reference(s) or source
Escherichia coli JM109	Cloning host for pUC19ery and its derivatives	Promega
Lactobacillus plantarum WCFS1	Wild-type: single colony isolate from human saliva isolate NCIMB8826	(17)
Lactobacillus plantarum WCFS1::pLAMA-K28	*L. plantarum* WCFS1 with single chromosomal integration of pLAMA-K28	This work
Lactobacillus plantarum MSΔlamA	DCO mutant in *L. plantarum* WCFS1 with *lamA* replaced by *lamA*::tag gene replacement cassette	This work
Lactobacillus plantarum NCIMB8826 Int-1	Em^R , stable *nisRK* integrant in the $tRNA^{Ser}$ locus, after transformation with pMEC10	(41)
Lactobacillus plantarum MSI8048	Em^R, Cm^R, *L. plantarum* NCIMB8826 Int-1 containing pNZ8048	This work
Lactobacillus plantarum MSI011	Em^R, Cm^R , *L. plantarum* NCIMB8826 Int-1 containing pNICE011	This work
pUC19ery	Amp^R, Em^R, 3.8 kb derivative of of pUC19 containing 1.1 kb *Hin*PI fragment of pIL253 carrying the Ery^R gene	(56)
pLAMA-K28	pUC19ery vector carrying *lamA*::tag gene replacement cassette	This work
pNZ8048	A derivative of the pNZ8030 series containing P_{nisA}, a MCS and T_{pepN}. Nisin Controlled Expression vector, Cm^R	(9, 41)
pNICE011	*lamBD* translationally fused (*Nco*I) to pNZ8048, Cm^R	This work
pBACe3.6	Bacterial Artificial Chromosome plasmid	

[a] Em^R, erythromycin resistant; Cm^R, chloramphenicol resistant; Amp^R, ampicillin resistant; DCO, double crossover; MCS, multiple cloning site

Table 4.2 Primers used in this study and their relevant characteristics

Primer	**Sequence (5'-3')** [a]
WCFS-lamA-*Nde*I	cagtgatt**catatg**TTGACCGGATGTTATCCAG
WCFS-lamA-*Eco*RI	cat**gaattc**ATTGGACAAGCACTTGCGACG
WCFS-lamA-*Kpn*I	acg**ggtacc**TTAGTAATGATTTGCCG
WCFS-lamA-*Bam*HI	aat**ggatcc**AGATCATTTGGCGAGTTTAG
pBAC-SacB-*Eco*RI	cgt**gaattc**TACACTGACTATTCCG
pBAC-SacB-*Kpn*I	tca**ggtacc**GTGTTTGAAGTGATCAGC
Lam-*Nde*I-junction-1	GTTAGGCCAAGTTACTTTGC
Lam-*Bam*HI-junction-4	CTACGCTTATTGGTACGAGC
SacB-control-2	CATCGATAAACTGCTGAACG
SacB-control-3	CGATGCTGAGTTAGCGAACG
RR-lamA-F	GCAGGCGACTTCCTTGAAAC
RR-lamA-R	GAAGCGAGTATCTCAGAATG
LamBD-NICE-*Kpn*I	CTA**GGTACC**TTATTTACGTAGTTCGTC
LamBD-NICE-*Nco*I	GGAGTGGG***CC*****ATG****G**AAAAGCCAGAAC
pNZ8048-F	GTTAGATACAATGATTTCGTTCG (left of MCS)
pNZ8048-R	CAATTGAACGTTTCAAGCCTTGG (right of MCS)
Lp1197-2F [b]	GACAAGCACGACCAACTCACA
Lp1197-2R [b]	TAAGCCGACAACTAGCCCAATCAA
Lp1204-1F [b]	ACGTCATCCATTTCGCTTTTT
Lp1204-1R [b]	GTCTCATTCCACGCATCTCTGT
Lp3581-2F [b]	GATTGGCCTATTTGTCATT
Lp3581-2R [b]	GAGCTTCGATTTCATTCA

[a] Capitals indicate nucleotides homologous to genome sequence; bold type characters indicate restriction enzyme recognition sequence; lower case characters indicate non-homologous nucleotides. Mismatches are in italics; start codon of LamBD is underlined.

[b] Primers used for real-time RT-PCR.

Adherence assays

Adherence-phenotype characterization and cell-aggregation were initially performed with strains grown in polystyrene petri-dishes (6 cm diameter) containing glass cover-slides and 5 ml MRS as has been described by Merritt et al. (33). For quantitative measurements strains were grown in 1 ml MRS in 24-well plates (TPP, Switzerland), with glass cover-slides (14 mm diameter; Menzel-Glazer) on the bottom of the wells. Wells containing medium only were used as blanks and a separate plate was used for optical density measurements. Cells were grown for 24 or 48 h at 30˚C after which medium was removed. Wells were washed twice with PBS to remove loosely attached cells and the remaining adhered cells were air-dried for 10 min. For staining 0.5 ml crystal violet (0.1% in deionized water) was added and incubated 30 min at 22˚C. Wells were washed three times with deionized water and the stained attached cells removed by dissolving them in 1 ml 96% ethanol. Absorbance was measured at 595 nm, with 6 replicates for each strain. Experiments were performed in independent triplicates and the statistical significance of differences was calculated using the Student's *t* test (two-tailed, two-sample equal variance).

Array design

For transcription profiling clone-based DNA microarrays were used, that were based on clones derived from the genomic library that was previously constructed for genome sequencing of *L. plantarum* WCFS1 (27). In total, 3692 genomic fragments were PCR-amplified from the genomic library using Supertaq (SphaeroQ, Leiden, the Netherlands), and vector-derived universal forward and reverse primers with 5'-C6 aminolinkers to facilitate cross-linking to the aldehyde-coated glass slides. The resulting amplicons had an average size of 1.2 kb, covered 80.8% of the genome, and represented 2683 of the 3052 annotated genes (88%). They were purified by ethanol precipitation and dissolved in 3xSSC. Subsequently, the purified amplicons were arrayed in a controlled atmosphere on CSS-100 silyated aldehyde glass slides with quill pins (Telechem, SMP3, USA) in a SDDC 2 Eurogridder (ESI, Toronto, Canada). Afterwards the slides were dried and blocked with borohydride.

cDNA preparation, fluorescent labelling and hybridisation

Differential transcript levels were determined by two-colour (Cy5 and Cy3) fluorescent hybridizations of the corresponding cDNAs on the clone-based DNA

microarray. Cy5/Cy3 dye-swaps were performed for the wild-type and *lamA*-mutant cDNA-samples. Labelled cDNAs were prepared according to protocols from Stanford University (The Brown Lab: http://cmgm.stanford.edu/pbrown/protocols/index.html) using random hexamers for reverse transcription. Total RNA (25 μg) was labeled during reverse transcription by incorporation of FluoroLink Cy3 or Cy5-labelled dUTP (Amersham Biosciences) using a reverse transcription kit (Gibco BRL). The reaction was stopped by adding 1N NaOH and neutralized with 1M HCL. Unincorporated dyes were removed from labeled fragments by using Autoseq G50 columns (Amersham Biosciences). Slides were prehybridized for 45 min at 42°C in 20 ml prehybridization solution (1% BSA, 5x SCC and 0.1% SDS, filtered), washed in filtered deionized water and dried. Co-hybridization of Cy5 and Cy3 labeled cDNA-probes was performed overnight at 42ºC in Easyhyb buffer (Roche) according to the manufacturer's protocol. The slides were washed twice in 1xSSC and 0,2% SDS, once in 0.5x SSC and twice in 0.2x SSC at 37°C. The slides were dried before scanning.

Microarray scanning and data analysis

Slides were scanned on a ScanArray Express 4000 scanner (Perkin Elmer) and image analysis and processing performed using the ImaGene version4.2 software-package (BioDiscovery). The criteria for flagging spots were as follows: (i) empty spots threshold 2.0, (ii) poor spots treshold 0.4, and (iii) negative spots. Routinely over 80% of all spots passed these quality criteria. Raw data were stored in BASE (48). Flagged data were discarded and the remaining, high-quality spot data were normalized using a LOWESS fit on M-A transformed data (64). To calculate a regulatory ratio for each gene, as far as the genes were represented by clones on the microarray, a weighted average of the M-values of all clones that overlapped with the gene of interest was calculated. The weight used for each clone was equal to the square of the overlap between gene and clone divided by the total length of the gene. Consequently, this method weighs small overlapping fragments less than proportional compared to larger overlapping fragments. Statistical analysis was performed with the statistical software program R (19) using the ANOVA model-fitting package maanova (24). Significant effects due to mutation (2 levels), growth-phase (3 levels) and experiment (2 levels) and their interactions were observed at different levels of significance ($P<0.05$ and $P<0.01$). Clones displaying these effects were selected and

analyzed with the Eisen CLUSTER-program (http://rana.lbl.gov/EisenSoftware.htm). Hierarchical clustering was performed using average linkage clustering and clustered genes were visualized with the Eisen Treeview program (http://rana.lbl.gov/EisenSoftware.htm).

Scanning Electron Microscopy

Wild-type and *lamA* mutant cultures were grown for 24 or 48 h in 24-wells plates with 8 mm diameter glass slides (Menzel-Glaser) on the bottom, before sample-preparation. Nucleopore Polycarbonate membranes (Costar, Cambridge, USA) with 1 μm pores were incubated for 30 min in a 0.01% poly-L-lysine solution in 0.1M Tris/HCl buffer. Planktonic cells were spotted on poly-L-lysine coated polycarbonate membranes and glass-adhered cells treated as such on the glass-slides. Bacteria were fixed for 30 min in 3% glutardialdehyde, membranes and glass slides washed 3 times with deionized water, dehydrated with ethanol, using 30%, 50%, 70%, 90% and finally 3x 100% ethanol and critical-point dried by the CO_2 method (Balzers CPD 020, Balzers Union, Liechtenstein). Dried membranes and glass slides were mounted on sample holders by carbon-adhesive tabs (Electron Microscopy Sciences, Washington, USA). Sample holders were positioned inside a sputter coater (JEOL JFS 1200 Fine Coater) and samples sputter coated with 10 nm gold and analyzed in a scanning electron microscope (JEOL JSM-5600 LV) at 3 kV. Images were recorded digitally.

Overexpression of LamBD using the NIsin Controlled Expression (NICE) system

The *lamBD* genes were amplified by PCR with primers LamBD-NICE-*Nco*I with LamBD-NICE-*Kpn*I and proofreading Platinum *Pfx* DNA polymerase (Invitrogen), using *L. plantarum* WCFS1 genomic DNA as a template. The PCR-product was translationally fused (*Nco*I-*Kpn*I) to the NICE-vector pNZ8048 (9, 41), after digesting primers and vector with *Nco*I and *Kpn*I, and cloned into the intermediate host *E. coli* JM109 by heat shock transformation. This plasmid, designated pNICE011, was subsequently cloned by electroporation into *L. plantarum* NCIMB8826 Int-1, carrying chromosomal integrations of the genes for the nisin response regulator *nisR* and histidine kinase *nisK* (41). Transformants were selected on MRS agar containing 5 μg ml^{-1} erythromycin and 10 μg ml^{-1} chloramphenicol and

analyzed by colony PCR using the pNZ8048 multiple-cloning-site flanking primers pNZ8048-F and pNZ8048-R. The NCIMB8826 Int-1 strain, carrying pNICE011, was designated MSI011, and the strain carrying the empty vector pNZ8048 was designated MSI8048.

Strain MSI011 was grown to an OD_{600} of 3.0 in a chemically defined medium (CDM) supplemented with 1% glucose and 10 μg ml^{-1} chloramphenicol (25). CDM was used to reduce the amounts of interfering proteinaceous impurities to improve analysis of overproduced peptides. The culture was 1:20 diluted to an OD_{600} of 0.15 in 10 ml fresh CDM containing 5 μg ml^{-1} chloramphenicol and incubated for 3 h at 37˚C. Nisin (ICN Biomedicals) was added to final concentrations of 0, 1.0, 5.0, 10.0, 20, or 60 ng ml^{-1}, and the cultures incubated for another 3 h at 37˚C.

LC/MS analysis of culture supernatant

Culture supernatants were prepared by centrifugation of cultures for 15 min at 6,500 x *g* at 4˚C. The supernatants were loaded onto a Sep-pak C_{18} cartridge column (100 mg; Waters Co., Milford Mass.), washed with 10 ml of 10% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and eluted with 5 ml of 60% acetonitrile containing 0.1% TFA. Eluates were dried by evaporation using a Speedvac concentrator, redissolved in 160 μl of 10% acetonitrile containing 0.05% TFA and 80 μl of these solutions were injected into a LC/MS device (LC: Agilent HP1100, column: Agilent Zorbax Eclipse XDB-C18, 2.1 x 50 mm, MS: JEOL Accutof T100LC, Tokyo, Japan). The column was eluted at a flow rate of 0.2 ml/min at 30ºC with a linear gradient of acetonitrile (10% to 36% in 40 min) in 0.05% TFA aqueous solution. The column eluates were directly loaded into the ESI-TOF mass spectrometer. Mass analyses were performed under the following conditions: positive polarity, capillary temperature 250°C, needle voltage 2.0 kV, orifice voltage 75 V for strain MSI011 or 35V for the WCFS1 wild-type and MSΔlamA strains and ring lens voltage 15 V. After scanning for molecular ions derived from column eluates in the *m/z* range of 100 to 2,000, extracted ion chromatograms were plotted with detector counts at the indicated *m/z*, with a window of one mass unit.

Chemical analysis of LamD558

LamD558 was purified from the Sep-pak purified culture supernatants of strain MSI011 by the same LC device as used for the LC/MS analysis described above. Amino acid sequence analysis was performed with the purified LamD558 (0.5 µg) by a peptide sequencer (Shimazu PSQ-21). For alkaline hydrolysis, 10 µl of 1M KOH was added to the purified LamD558 (0.25 µg / 90 µl of 10% acetonitrile containing 0.05% TFA) and incubated overnight at 37ºC. Subsequently 5 µl of acetic acid was added to neutralize the reaction solution. Of this solution, 50 µl was subjected to LC/MS, for which the same conditions were used as for the analysis of culture supernatant described above, except that the collision induced dissociation (CID) spectrum was measured at the orifice voltage of 95 V. As a control, the synthetic linear five-amino acid peptide, Cys-Val-Gly-Ile-Trp, was subjected to the same LC/CID-MS analysis (see below).

Chemical synthesis of LamD558

The linear peptide, Cys-Gly-Val-Ile-Trp, was manually synthesized by the solid-phase method using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy as previously described (35). *N*-α-Fmoc-*S*-trityl-L-cystein (Novabiochem, Merck Limited, Tokyo, Japan) was used for the synthesis of the protected peptide and deblocking was done by stirring the resin carrying the synthetic peptide in a TFA/phenol/H_20/ethanedithiol/triisopropylsilane mixture (86:5:5:3:1) for 1.5 h at room temperature. Two milligrams of the crude peptide was dissolved in 0.8 ml of *N*, *N*-dimethylacetamide and (benzotriazole-1-yl)oxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBop) (Novabiochem; 4 mg) and dimethlyaminopyridine (4 mg) were added to the solution. The reaction mixture was stirred at room temperature for 6 h under N_2 gas and then added to 32 ml of ice-cold water. The solution was loaded onto a Sep-pak C_{18} cartridge column (Waters, 1ml), the column washed with 10 ml of 15% acetonitrile containing 0.1% TFA, and the cyclized peptide eluted with 5 ml of 60% acetonitrile containing 0.1% TFA. The obtained peptide was further purified by reverse-phase HPLC using a linear gradient of acetonitrile in 0.1% TFA on a Pegasil ODS column (0.46 x 15 cm, Senshu Kagaku, Tokyo, Japan).

Real-time reverse transcription PCR

A small number of open reading frames (ORFs) was chosen for quantitative real-time reverse transcription (RT) PCR confirmation of data obtained from microarray analysis at early-, mid- and late-logarithmic growth-phases, as described above. To analyze the functionality of LamD558, synthetic peptide was added at 100 and 500 nM to early log-phase wild-type cultures (OD_{600} of 0.5) and RNA isolated at 0.5, 1.0 and 2.0 h post-induction, as described above. Induction of gene-expression of selected ORFs was determined by real-time RT-PCR quantification. cDNA was generated from total RNA after reverse transcription with random hexamers, according to the manufacturer's instructions (Invitrogen). Real-time PCR was performed on cDNA using gene-specific primers (Table 4.2) with the BioRad Sybr Green kit in a BioRad I-Cycler. Measurements were performed in triplicate and gene-specific mRNA amounts normalized against the 16S rRNA amount in the sample.

Results

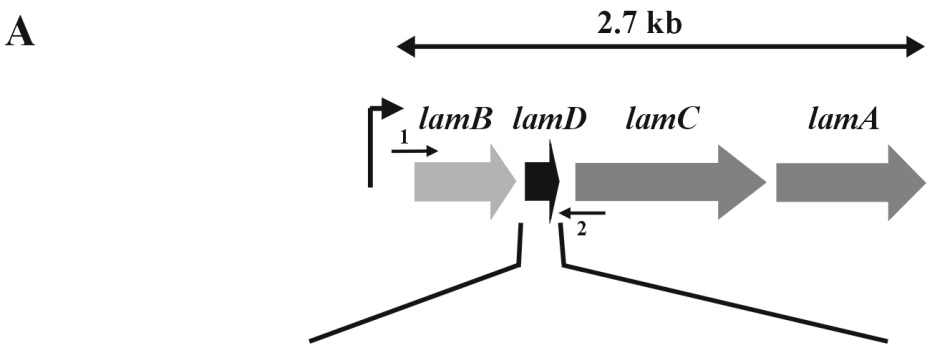
The *lamBDCA* operon of *L. plantarum*

The *L. plantarum* WCFS1 genome encodes a two-component regulatory system that is homologous to the *agr*-system of *S. aureus* and was therefore designated *lam* for *Lactobacillus agr*-like module (lp_3580 to lp_3582 on the *L. plantarum* WCFS1 genome-project website http://www.cmbi.kun.nl/lactobacillus (27)). The *lamBDCA* genes (Fig. 4.1A) are predicted to code for a histidine protein kinase LamC, showing 24% identity to the *S. aureus* AgrC protein, and its cognate response regulator LamA, showing 37% identity to the *S. aureus* AgrA protein. It is also predicted to encode LamB, a protein showing 30% identity to the *S. aureus* AgrD-processing protein AgrB. Alignment of the putative *lamD*-encoded peptide (located between *lamB* and *lamC*) with staphylococcal AgrD peptide-sequences, showed that it contained the conserved amino acids found in AgrD peptides (Fig. 4.1B), The *lamD* gene therefore appeared to encode an AIP precursor peptide. The amino acids shown in bold type in Fig. 4.1B (LVMCCVGIW) were predicted to comprise the AIP sequence that could be cleaved off from the precursor peptide LamD, and modified to a cyclic thiolactone peptide, in analogy with the *agr*- and *fsr*-systems in staphylococci (20, 40) and *E. faecalis*, respectively (34). The expression of

lamBDCA during growth was analyzed by Northern blot analysis and appeared to be expressed as a single 2.7-kb transcript, comprising the complete *lamBDCA*-operon. Expression was detectable at lower levels in early growth phases with a clear 2.5-3.0 fold increase at 5 h post-inoculation (mid-exponential phase) that appeared to be maintained until late-log and early stationary phase (Fig. 4.2), suggesting growth-phase dependent transcription regulation.

Effect of *lamA* deletion on adherence

A *lamA* gene replacement mutant was constructed, as deletion of this regulator should exclude cross-talk to the *lam*-system by other regulatory circuits. Initial experiments showed no differences in growth-rate and cell- or colony morphology between wild-type and *lamA*-mutant (data not shown). As *agr*-systems in staphylococci are involved in biofilm formation (65), the ability to adhere to different surfaces as well as cell-cell aggregation was investigated for the wild-type and *lamA*-mutant. Both wild-type and *lamA*-mutant did not form planktonic cell-aggregates nor adhere to polystyrene (data not shown). However the mutant showed significantly decreased glass-adherence compared to the wild-type after both 24 and 48 h, as determined in a quantitative assay ($P<0.001$; Fig. 4.3). Scanning electron microscopy analysis of wild-type and *lamA*-mutant confirmed that the mutant did not form a distinct biofilm on glass slides in contrast to the wild-type. The wild-type formed a dense layer with protruding structures, while the mutant only formed a thin layer on the slides (Fig. 4.4). The cell surfaces of both strains showed no visible differences at larger magnifications (data not shown).



B

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Sau-I          MNTLFNLFF-DFITGILKNIGNIAA YSTCDFIM DEVEVPKELTQLHE------- 46
Sau-IV         MNTLYKSFF-DFITGVLKNIGNVAS YSTCYFIM DEVEIPKELTQL--------- 44
Sau-III        MKKLLNKVI-ELLVDFFNSIGYRAAY INCDFLL DEAEVPKELTQLHE------- 46
Slug-I         MNLLSGLFT-KGISAIFEFIGNFSAQ DICNAYF DEPEVPQELIDLQRKQLIESV 53
Slug-II        MNLLSGLFT-KGISVIFEFIGNFSVQ DMCNGYF DEPEVPQELIDLHRN------ 47
Sepi-I         MNLLGGLLL-KLFSNFMAVIGNAAK YNPCASYL DEPQVPEELTKLDE------- 46
Sepi-III       MNLLGGLLL-KIFSNFMAVIGNASK YNPCSNYL DEPQVPEELTKLDE------- 46
Sepi-II        MENIFNLFI-KFFTTILEFIGTVAG DSVCASYF DEPEVPEELTKLYE------- 46
Sau-II         MNTLVNMFF-DFIIKLAKAIGIVG GVNACSSLF DEPKVPAELTNLYDK------ 47
LplantWCFS1    ---MKQKMY-EAIAHLFKYVGAKQ LVMCCVGIW FETKIPDELRK---------- 40
                  :   . . : .   :*       *      * ::* ** .
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Figure 4.1 (A) Schematic representation of the gene organization of the *lamBDCA* operon, as deduced from the *Lactobacillus plantarum* WCFS1 genome project (http://www.cmbi.kun.nl/lactobacillus). The position and direction of NICE cloning primer LamBD-NICE-*Nco*I (arrow 1) and NICE cloning primer LamBD-NICE-*Kpn*I (arrow 2) are shown. (B) Alignment of the WCFS1 LamD prepeptide with staphylococcal AgrD prepeptide sequences. The numbers of amino acids in the prepeptides are shown to the right of the sequences. Sau-I-IV: *S. aureus* pherotypes I-IV; Sepi-I-III: *S. epidermidis* pherotypes I-III; Slug-I-II: *S. lugdunensis* pherotypes I-II; LplantWCFS1: *L. plantarum* WCFS1. Amino acids of determined or predicted AIP sequences are shown in bold type. Asterisks indicate conserved amino acids in all sequences; colons indicate conserved amino acid substitutions and dots indicate semi-conserved amino acid substitutions according to physico-chemical criteria. Gaps introduced to maximize alignment are indicated by dashes.

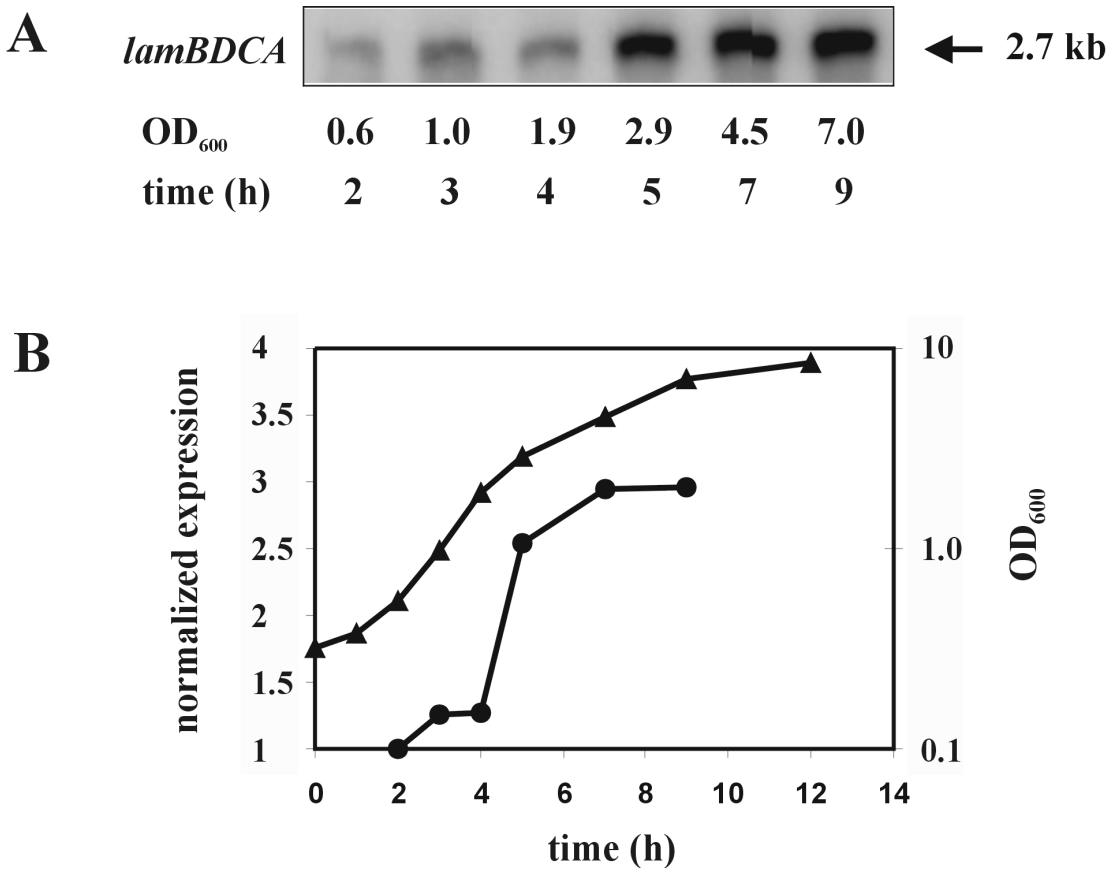

Figure 4.2 Northern blot analysis of temporal expression of the *lam*-cluster. (A) Expression of *lam* during growth using a *lamC* internal probe. Similar results were obtained with either the *lamB* or *lamA* probes (B) Graphical representation of *lam*-expression levels on Northern blots normalized against the 16S rRNA expression level. Triangles: OD_{600} of MRS culture; circles: normalized *lam*-expression.

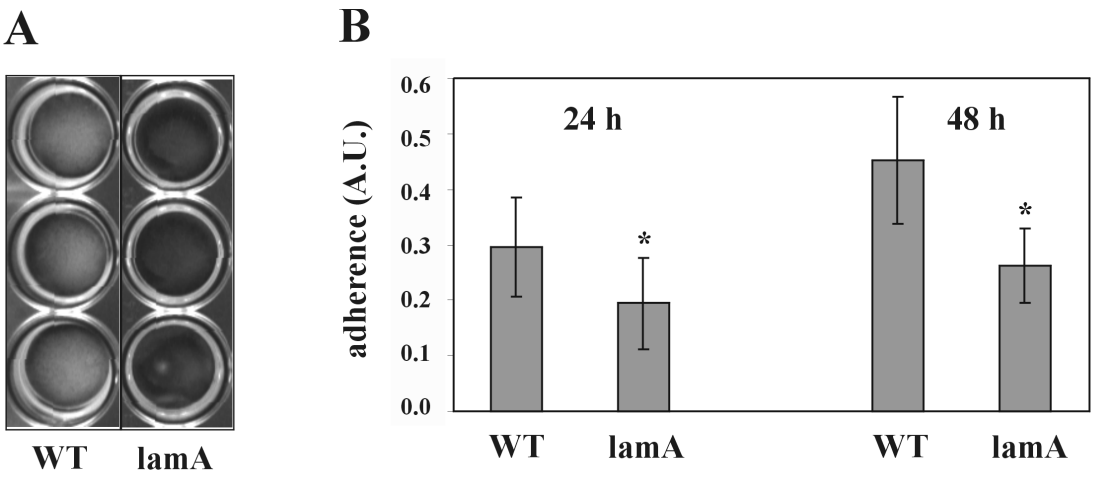

Figure 4.3 Quantification of glass-adherence in 24-well plates of the *L. plantarum* WCFS1 wild-type (WT) and MSΔlamA (lamA) strains grown in MRS. (A) Picture showing glass-adhered cells grown in MRS in 24-wells plates with glass cover-slides after the phosphate-buffered saline wash-step. (B) Quantification of crystal violet-stained glass-adhered cells in 24-well plate assay. Adherence of wild-type (WT) and MSΔlamA (lamA) strains was measured in arbitrary units (A.U.). Means ± standard errors (error bars) of three 24-well plates, with six wells measured per plate are shown. Values that were statistically significant different from the WT value (P<0.001) are indicated by an asterisk. Statistical comparisons were done using the Student's *t* test (two-tailed, two-sample equal variance).

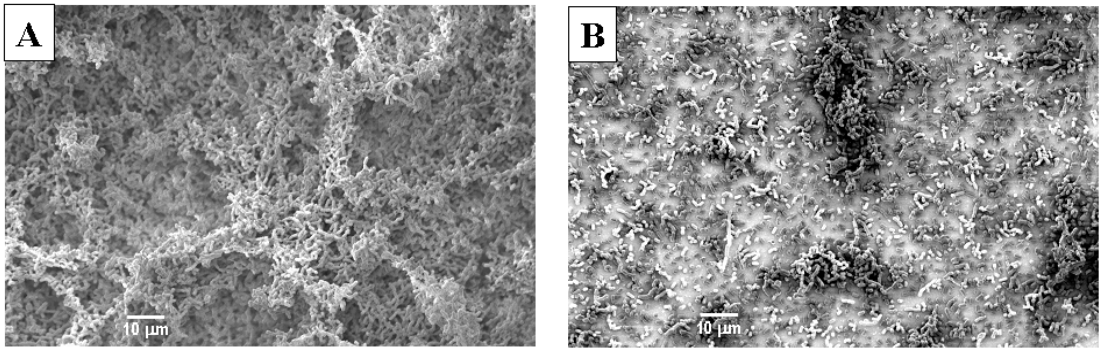

Figure 4.4 Scanning electron micrographs of biofilm structures of glass-adhered cells of the wild-type (A) and *lamA* mutant (B) respectively. Magnification, x1,000.

DNA microarray transcriptional analysis of *lamA*-mutant versus wild-type

To study the global effects of the *lamA* deletion, transcription profiles in early-, mid- and late-logarithmic growth phases (OD_{600} of 0.6, 2.0 and 4.8, respectively) were determined for the wild-type and *lamA*-mutant strains, using a clone-based microarray. After scanning, clones showing significant differences in gene expression for the mutation-effect were selected using ANOVA statistical analysis and their transcription profiles hierarchically clustered. Clones displaying a significant mutation-effect ($P<0.05$) independently and consistently identified several genes with a difference in expression profile for *lamA*-mutant compared to wild-type (see Table 4.3). Hierarchical cluster-analysis of the transcription-profiles per clone identified four distinct clusters of responding genes (Table 4.3). Cluster 1 contained several sugar utilization genes that were strongly down-regulated in late logarithmic phase only (e.g. sucrose, cellobiose and trehalose PTS genes lp_0185, lp_0436 and lp_0264, respectively), Cluster 2 encompassed constitutively down-regulated genes, with the highest effect in mid-logarithmic growth phase, coding for the *lamBDCA*-locus (lp_3580 to lp_3582) and several flanking genes as well as genes encoding integral membrane proteins (e.g. lp_0926, lp_3575 and lp_3577). Cluster 3 encompassed constitutively up-regulated genes, with the highest effect in early-logarithmic growth phase, coding for a complete surface polysaccharide locus (*cps2*: lp_1197 to lp_1211). Cluster 4 finally, contained genes that mainly showed up-regulation in mid-logarithmic phase, with most gene-functions related to pyrimidine-biosynthesis (lp_2698 to lp_2703). The results obtained by microarray analysis were confirmed by quantitative real-time RT-PCR, using gene-specific primers for gene *lamC* (lp_3581), and genes lp_1197 and lp_1204 of *cps2* (see Table 4.3).

Table 4.3 Hierarchical clusters of genes showing significant differences in gene expression after ANOVA statistical analysis in the *lamA* mutant versus wild-type strain during early-, mid- and late- logarithmic growth.

Cluster and ORF[a]	Gene[b]	Description	No. of clones	Change (fold)[c]		
				E	M[d]	L
Cluster 1						
lp_0184	*sacK1*	Fructokinase	2	1.1	0.0	-12.1
lp_0185	*pts1BCA*	Sucrose PTS, EIIBCA[e]	3	1.0	-0.2	-13.6
lp_0187	*sacA*	β-Fructofuranosidase	1	1.1	-0.3	-18.5
lp_0188	*sacR*	Sucrose operon repressor	1	0.3	1.6	-14.6
lp_0189	*agl2*	α-Glucosidase	1	0.3	1.6	-14.6
lp_0263	*treA*	α, α-Phosphotrehalase	1	-2.2	0.4	-15.3
lp_0264	*pts4ABC*	PTS, trehalose-specific IIBC component	2	-1.9	0.4	-13.9
lp_0435		Transcription regulator, GntR-family	1	1.8	0.4	-8.2
lp_0436	*pts7C*	Cellobiose PTS, EIIC	1	1.8	0.4	-8.2
lp_3480	*galT*	UTP-hexose-1-phosphate uridylyltransferase	1	1.1	1.4	-10.6
lp_3481	*galE4*	UDP-glucose 4-epimerase	1	1.1	1.4	-10.6
lp_3482	*galK*	Galactokinase	1	1.4	-0.2	-21.9
Cluster 2						
lp_0525	*kup1*	Potassium uptake protein	1	-2.8	-3.9	-2.7
lp_0526	*carB*	Carbamoyl-phosphate synthase, large subunit	1	-2.8	-3.9	-2.7
lp_0683		Prophage P1 protein 60	1	0.5	-4.5	-3.7
lp_0684		Prophage P1 protein 61	1	0.5	-4.5	-3.7
lp_0925		Acyltransferase	1	-0.7	-4.0	-5.7
lp_0926		Integral membrane protein	1	-8.8	-11.0	-6.1
lp_0927		Hypothetical protein	2	-8.0	-10.4	-6.7
lp_0928		Hypothetical protein	2	-8.0	-10.4	-6.7
lp_0929	*asp1*	Alkaline shock protein	2	-8.0	-10.4	-6.7
lp_0930	*asp2*	Alkaline shock protein	2	-8.0	-10.4	-6.7
lp_0931	*hpaG*	2-Hydroxyhepta-2,4-diene-1,7-dioateisomerase / 5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioatedecarboxylase (putative)	1	-7.1	-9.8	-7.3
lp_2658		Glycosyltransferase (putative)	2	-4.5	-3.8	-3.2
lp_2743		ABC transporter, ATP-binding protein	1	-7.6	-10.5	-8.0
lp_2744		ABC transporter, permease protein	1	-7.6	-10.5	-8.0
lp_3045		Short-chain dehydrogenase/oxidoreductase	1	-6.6	-4.8	-3.1
lp_3047		Hypothetical protein	1	-6.6	-4.8	-3.1
lp_3575		Integral membrane protein	1	-6.7	-11.0	-7.5
lp_3577		Integral membrane protein	2	-4.6	-3.8	-3.7
lp_3578	*kat*	Catalase	4	-4.4	-3.8	-3.6
lp_3580	*lamA*	Response regulator; homolog of accesory gene regulator protein A	3	-2.7	-4.7	-2.8
lp_3581	*lamC*	Histidine protein kinase; sensor protein	3	-2.7 (-2.3)	-4.7 (-2.8)	-2.8 (-2.7)
lp_3581a	*lamD*	Homologue of accesory gene regulator protein D, peptide pheromone precursor	1	-2.4	-4.7	-2.7
lp_3582	*lamB*	Homologue of accesory gene regulator protein B	1	-2.4	-4.7	-2.7
lp_3583	*clpL*	ATP-dependent Clp protease, ATP-binding subunit ClpL	3	-21.6	-27.6	-11.7
lp_3586	*lox*	Lactate oxidase	2	-24.5	-28.7	-22.7

Table 4.3 – *Continued*

Cluster and ORF[a]	Gene[b]	Description	No. of clones	Change (fold)[c]		
				E	M[d]	L
Cluster 3						
lp_1197	*cps2A*	Exopolysaccharide biosynthesis protein	1	40.5 (21.7)	16.6 (9.9)	19.0 (21.7)
lp_1198	*cps2B*	Exopolysaccharide biosynthesis protein; chain length determinator Wzz	2	28.9	16.6	16.1
lp_1199	*cps2C*	Exopolysaccharide biosynthesis protein	1	17.2	16.5	13.2
lp_1200	*galE2*	UDP-glucose 4-epimerase	3	26.6	14.3	18.5
lp_1201	*cps2E*	Priming glycosyltransferase	3	26.6	14.3	18.5
lp_1202	*cps2F*	Glycosyltransferase	2	37.0	12.5	13.3
lp_1203	*cps2G*	Glycosyltransferase	2	38.5	11.1	11.3
lp_1204	*cps2H*	Polysaccharide polymerase	2	28.0 (37.0)	7.8 (10.4)	8.1 (12.4)
lp_1205	*cps2I*	Repeat unit transporter	3	19.2	3.7	4.4
lp_1206	*cps2J*	Glycosyltransferase	1	18	2.6	3.6
lp_1207		Hypothetical protein	1	11.6	1.9	3.4
lp_1208		Hypothetical protein	1	11.6	1.9	3.4
lp_1210		Hypothetical protein	1	5.5	0.7	1.3
lp_1211		Hypothetical protein	1	5.5	0.7	1.3
Cluster 4						
lp_0254	*cysE*	Serine *O*-acetyltransferase	2	-0.1	5.5	5.4
lp_0255	*metC1*	Cystathionine beta-lyase	3	-0.4	5.8	5.4
lp_0256	*cysK*	Cysteine synthase	2	-0.6	5.9	5.7
lp_2371	*pyrP*	Uracil transport protein	1	0.6	13.2	0.7
lp_2684	*araT2*	Aromatic amino acid specific aminotransferase	2	0.6	0.8	19.0
lp_2685	*dapA2*	Dihydrodipicolinate synthase	2	0.6	0.8	19.0
lp_2698	*pyrF*	Orotidine-5'-phosphate decarboxylase	1	-0.4	34.6	-0.7
lp_2699	*pyrD*	Dihydroorotate oxidase	2	-0.6	46.6	-0.7
lp_2700	*pyrAB*	Carbamoyl-phosphate synthase, pyrimidine-specific, large chain	4	0	59.2	-0.4
lp_2701	*pyrAA*	Carbamoyl-phosphate synthase, pyrimidine-specific, small chain	4	0.6	69.9	-0.2
lp_2702	*pyrC*	Dihydroorotase	3	1.0	67.1	-0.3
lp_2703	*pyrB*	Aspartate carbamoyltransferase	1	1.0	11.3	0.0
lp_2704	*pyrR1*	Pyrimidine operon regulator	1	1.0	11.3	0.0

[a] Gene lp_number from the *L. plantarum* WCFS1 genome project (http://www.cmbi.kun.nl/lactobacillus).

[b] Previously reported gene name.

[c] Average change in the *lamA* mutant versus the wild-type strain during early (E), mid (M), and late (L) logarithmic growth. Average of four arrays unless specified otherwise. Average of all significant clones encoding the gene. The values in parentheses are the fold-changes as determined by real-time RT-PCR.

[d] Average of three arrays instead of four arrays.

[e] PTS, phosphotransferase system; EIIBCA, enzyme IIBCA.

Overexpression of LamBD using the NIsin Controlled Expression system (NICE)

To confirm the presence and elucidate the chemical structure of the predicted AgrD-like peptide, the *lamBD* genes were overexpressed in *L. plantarum* NCIMB8826 Int-1 using the NICE overproduction system (41). Overexpression was induced and culture supernatants were analyzed by liquid chromatography/mass spectrometer (LC/MS) analysis. Molecules eluted from a reverse-phase column were traced in the extracted ion chromatograms at the protonated molecular masses of possible LamD-derived peptide candidates. These candidates were predicted to be thiolactone peptides of four to ten residues within the amino acid stretch running from Leu-21 to Phe-30 of the LamD precursor peptide sequence, based on the alignments with staphylococcal AgrD precursor peptides (see Fig. 4.1B). Among those mass chromatograms, only one at a *m/z* value of 559 showed a clear peak at a retention time of 27 min in the detected ion-count, which suggested the presence of a five-residue thiolactone peptide from Cys-25 to Trp-29 (sequence CVGIW; Fig. 4.5A). Compared to the mass chromatogram of a non-induced culture supernatant, this peak clearly increased with increasing amounts of nisin (Fig. 4.5B). The peptide contained in this peak was designated LamD558, based on its molecular mass. The HPLC-fraction corresponding to this peak was collected and subjected to N-terminal amino acid sequencing on an automated peptide sequencer. This yielded the peptide-sequence X-Val-Gly-Ile-X, which was in agreement with the proposed thiolactone-structure in which Cys-1 was linked to Trp-5 via a thioester-bond. The determined accurate mass for this peptide was a *m/z* value of 559.2704, which was identical to the M+H value of 559.2703 that was calculated from the molecular formula of the predicted thiolactone peptide CVGIW. Alkaline-treatment of LamD558 increased the molecular weight by 18 mass units, suggesting that the thiolactone peptide was linearized by hydrolysis of the thioester bond (data not shown). Collision-induced-dissociation mass spectrometry of the alkaline-treated LamD558 produced fragment ions at *m/z* values of 260, 345 and 373, each corresponding to b3, a4 and b4 ions of the deduced peptide CVGIW, and the fragmentation pattern was identical to that of the chemically-synthesized five-residue linear peptide CVGIW (Fig. 4.6). Finally, we confirmed that the synthetic peptide possessing the proposed structure showed the same retention time in reverse-phase HPLC as LamD558 did (Fig. 4.7). From these results we conclude that *lamD* encodes a five-residue thiolactone peptide with the sequence CVGIW, which matches the predicted *lamD* precursor peptide sequence (see Figs. 4.1 and 4.8).

LamD558 could also be detected by LC/MS analysis in culture supernatants of the wild-type strain, as a function of time (Fig. 4.9). The amount of LamD558 increased markedly between 3 and 9 h post-inoculation, corresponding to the mid-log to late-log growth-phase. In a *lamA* deletion-strain, the detected amount of LamD558 was several times lower than that of the wild-type strain throughout the sampling period, which is in agreement with the autoregulatory function that was predicted for *lamBDCA* (Fig. 4.9).

Functionality of LamD558

To study the functionality of the identified LamD558 peptide, induction experiments were performed with early log-phase wild-type cultures using synthetic LamD558 peptide. mRNA amounts for genes *lamC* (lp_3581) and lp_1204 were quantified using real-time RT-PCR, at 0.5, 1.0 and 2.0 h post-induction. Induction with both 100 nM (concentration in mid-log culture supernatants) and 500 nM LamD558 showed no autoinducing effect for *lamC*, but in stead a non-anticipated moderate decrease (fold-change –1.1 to –2.2 at 100 nM, and –2.6 to –1.9 at 500 nM). For gene lp_1204 a moderate decrease in gene-expression was observed (fold-change –1.6 to –5.0 at 100 nM, and –4.8 to –2.3 at 500 nM), which was expected considering the microarray analysis that showed increased lp_1204 gene-expression in the *lamA*-mutant.

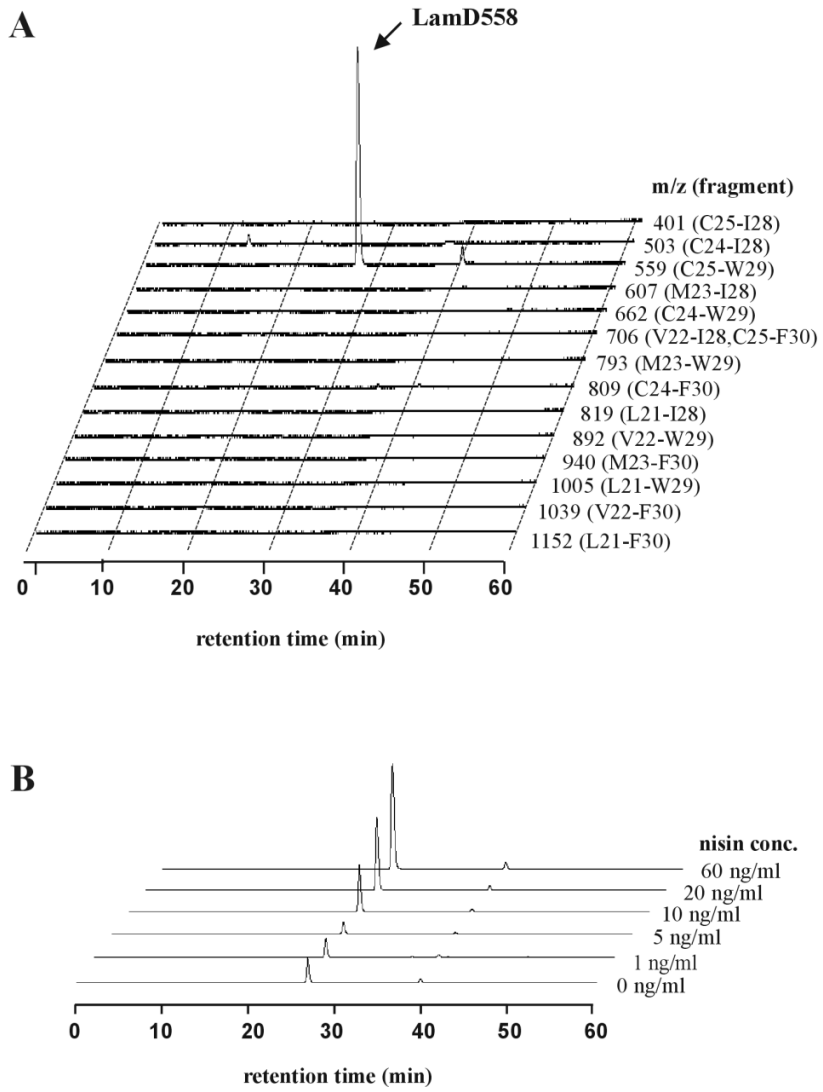

Figure 4.5 LC/MS analysis of culture supernatant of strain MSI011. The culture supernatant was prepared and analyzed by LC/MS as described in Materials and Methods. (A) Three-dimensional view of extracted ion chromatograms at different *m/z* values corresponding to possible LamD-derived peptide candidates. Twenty nanograms per milliliter of nisin was used for induction. Each *m/z* value used in the extracted ion chromatogram corresponds to a thiolactone peptide derived from the LamD-fragment indicated in parentheses. (B) Three-dimensional view of extracted ion chromatograms at a *m/z* value of 559 (C25-W29) with increasing concentrations (conc.) of nisin added as indicated.

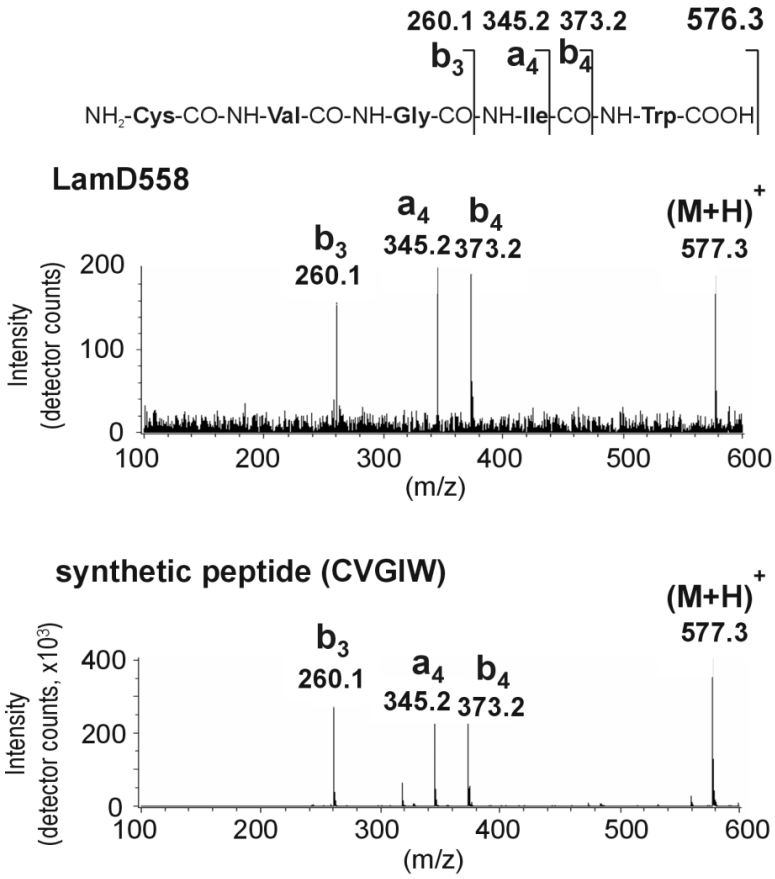

Figure 4.6 Collision-induced dissociation mass spectra of alkaline-treated LamD558 (top) and the synthetic linear CVGIW peptide (bottom). The alkaline-treated LamD558 and the synthetic pentapeptide were subjected to the LC/CID-MS analysis as described in Materials and Methods.

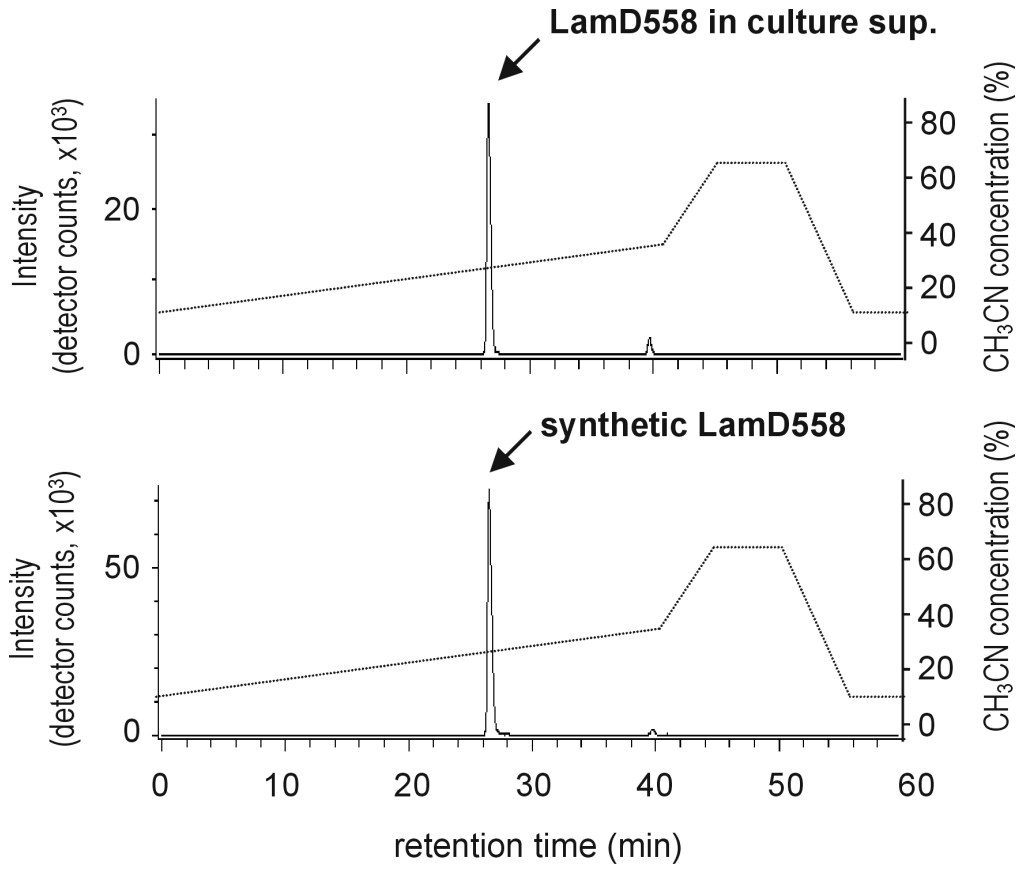

Figure 4.7 LC/MS analysis of LamD558 in culture supernatant (sup.) of strain MSI011 (top) and chemically-synthesized LamD558 (bottom). The extracted ion chromatogram at a *m/z* value of 559 was displayed for each analysis. The culture supernatant was prepared and LC/MS analysis performed as described in the legend to Figure 4.5A. The synthetic LamD558 was prepared as described in Materials and Methods.

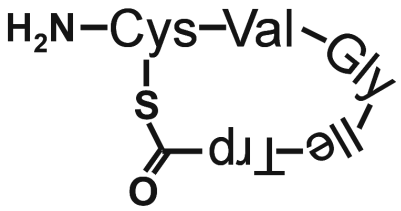

Figure 4.8 Structure of peptide LamD558.

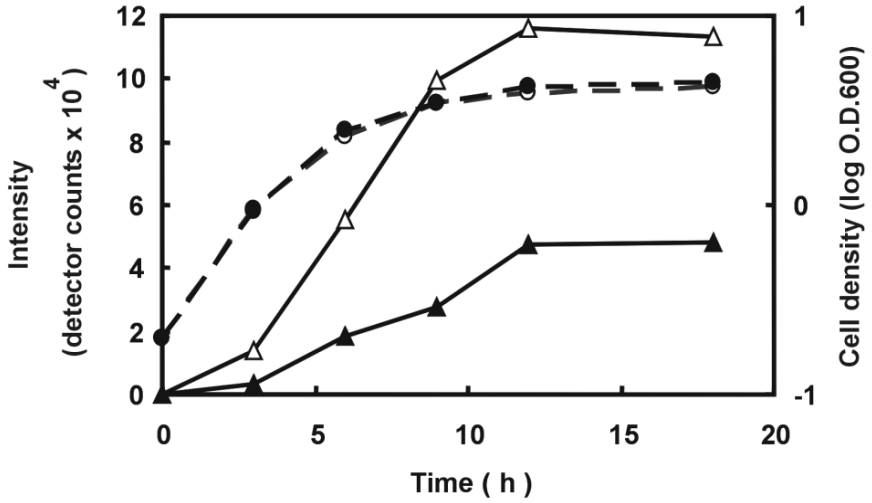

Figure 4.9 Time course of LamD558 production in culture filtrates of wild- type and *lamA*-mutant strain grown in CDM. Solid line shows LamD558 intensity (open triangle, wild-type strain; closed triangle, *lamA*-mutant strain). Broken line shows optical density of cultures at 600nm (log O.D.600) (open circle, wild-type strain; closed circle, *lamA*-mutant strain). Culture filtrates were collected and analyzed by LC/MS. Overnight cultures were inoculated into CDM to OD_{600} = 0.15 and cultured at 30˚C. Culture filtrates were collected at the indicated time and analyzed by LC/MS as described in Materials and Methods. The height of the LamD558 peak in each extracted ion chromatogram at a *m/z* value of 559 was plotted in the graph.

Discussion

The function of an *agr*-like two-component regulatory system of *Lactobacillus plantarum* WCFS1, designated *lamBDCA*, was studied by mutation analysis and a global transcriptional profiling approach. The *lamBDCA* genes were expressed in all growth phases with a clear increase at 5 h post-inoculation (mid-exponential phase) that remained until late-log to stationary phase, suggesting growth-phase dependent transcription regulation. In analogy, analysis of the homologous *agr* and *fsr* systems from *S. aureus* and *E. faecalis* OG1RF, respectively showed transcription initiation at mid-to late-exponential growth (45, 57). A *lamA* mutant was constructed and showed decreased attachment in a glass-adherence assay, which pointed to an involvement in regulation of cell-surface properties. The *lamA* mutant and wild-type strains showed similar growth-curves, which simplified global transcription analysis. Transcriptional profiling using microarrays showed altered transcription profiles of the *lamA* mutant compared to the wild-type strain for a limited number of loci, with several gene-clusters showing continuous up- or down-regulation for all growth-phases analyzed. The *lamBDCA*-genes were continuously down-regulated in the *lamA* mutant, confirming its analogy with an *agr*-like autoinducing system. Many of the *lamA*-affected genes appeared to be involved in cell surface properties, such as the up-regulated surface polysaccharide-biosynthesis genes and several down-regulated integral membrane proteins with unknown functions. Also UTP-hexose-1-phosphate uridylyltransferase, UDP-glucose-4-epimerase and galactokinase, that are involved in the production of activated glucose and galactose (51, 63) were down-regulated in the *lamA*-mutant, markedly in late-log phase. This was also found for an *agr*-null mutant in *S. aureus* (12). Several pyrimidine biosynthesis genes, which might be linked to UTP-biosynthesis from UMP (5), as well as an uracil-transport protein were up-regulated. The up-regulation of these genes could suggest increased cellular UTP-levels that might be necessary for and directed to surface polysaccharides biosynthesis via (UDP-)glucose, as the pathway via (UDP-)galactose is down-regulated while the surface polysaccharides biosynthesis genes are up-regulated (62). Finally, several genes mainly linked to sucrose, cellobiose and trehalose uptake and fructose-metabolism were down-regulated in late-log phase, which might indicate that *lamA* regulates transport of these sugars. At this stage, it is not clear whether the responding genes are under direct regulation of *lamA* or are the result of secondary effects. The

application of sequence pattern recognition software like MEME (3) did not allow the identification of specific conserved potential *cis*-acting elements in the upstream regions of the genes affected by the *lamA* mutation. This might suggest that the majority of the identified responses represent secondary regulatory effects rather than primary *lamA*-regulated effects. A similar response was found for an *agr*-null mutant in *S. aureus* (12).

The *lamA* deletion stimulates surface polysaccharide expression (up-regulation of *cps2*) in *L. plantarum* WCFS1, while it reduces adherence to a glass-surface. At first sight these findings may seem to contradict the results of other studies, which show that exopolysaccharide production is necessary for biofilm formation (53). Reports on *agr*-systems in *S. aureus* and *S. epidermidis* have suggested a positive influence of *agr* on biofilm-formation (60, 61). However, recent studies by Yarwood et al. (65) indicate that depending on the environmental conditions the biofilm development can either be inhibited or enhanced. This could indicate that other factors and regulatory systems might be involved in these phenotypes. If the surface polysaccharide operon codes for capsular (membrane-associated) rather than excreted exopolysaccharides, shielding of adhesion factors by capsular polysaccharides could explain our findings. Such shielding has also been observed in *S. aureus* and might explain our observations, with respect to the reduced glass-adherence of the *lamA* mutant (43). Finally, there are reports on surface characteristics being dependent on the relative expression of different lipopolysaccharides in *Pseudomonas aeruginosa,* which influence adherence properties to hydrophilic and hydrophobic surfaces (31). The detectable production of a biofilm may therefore be due to a delicate balance between different polysaccharides produced by *L. plantarum*, keeping in mind that the *L. plantarum* genome contains four different surface polysaccharides gene clusters, *cps1* (lp_1176 to lp_1190), the effected *cps2* (lp_1197 to lp_1211), *cps3* (lp_1215 to lp_1227), and *cps4* (lp_2099 to lp_2108) (27).

The number of ORFs showing altered gene-expression in the *lamA* mutant was 65 out of 3052 predicted ORFs (about 2%), which is lower then the 138 genes (about 5%) that were reported for an *agr*-null mutant in *S. aureus* (12). In the latter study, microarray comparison of wild-type versus an *agr*-null mutant in *S. aureus* showed that *agr* up-regulates *agrBDCA* and many recognized *agr* up-regulated genes coding for extracellular accessory proteins involved in virulence. In addition, several *agr*-regulated genes were identified that suggested a coupling of the *agr*-system to a

global cellular response, such as the up-regulated pyrimidine biosynthesis genes (*pyrAA*, *pyrR*, *arcB*, *arcS*) and the UTP-glucose-1-phosphate uridylyltransferase gene, and down-regulated genes coding for alpha-glucosidase (*treA*) and a GntR-type transcriptional regulator (12). Strikingly, similar responding genes were also found for the non-pathogenic *L. plantarum* WCFS1 *lamA*-mutant, suggesting that *agrBDCA* and *lamBDCA* might influence regulation of these genes via comparable molecular mechanisms (see Table 4.3).

To identify the presence of a secreted *agr*-like AIP and elucidate its structure, homologous overexpression of the *lamBD* genes, using the NICE system, was combined with LC/MS analysis of culture supernatants. This strategy allowed for the detection of secreted peptides in culture supernatants as well as a direct MS-identification strategy by multistage mass spectrometry, which had proven to be successful for rapid detection of staphylococcal AIPs (23). *L. plantarum* strains carrying the *lamBD* overexpression construct under the control of an autoinducing promoter were cultured in chemically defined medium, to reduce the amount of impurities that could generate high peptide backgrounds in chromatography, and also to reduce the ionization efficiency of the overexpressed target-peptide during mass spectrometry. Using these methods, overexpression of the *lamBD* genes identified an overproduced *agr*-like peptide with sequence CVGIW, which matched within the predicted LamD peptide, and contained a cyclic thiolactone bond from C to W (Figs. 4.1 and 4.8). This is in analogy with structural data obtained for autoinducing peptides in staphylococci and is the first finding of an *agr*-like peptide in lactobacilli. LamD558 consists of a five-amino-acid ring similar to known staphylococcal autoinducing peptides, but it lacks the N-terminal tail moiety consisting of two to four amino acids that is found in staphylococcal AIPs (40). In staphylococci this tail moiety is essential for functional interaction with the sensor AgrC (32). LamD558 is probably formed during processing and transport of the precursor peptide LamD by the AgrB-homologous protein LamB, although the mechanism has not yet been elucidated. A precursor peptide-processing mechanism has however been proposed for processing of AgrD by the AgrB protein in *S. aureus* (46) and GBAP by the FsrB protein in *E. faecalis* (37), involving a histidine and cysteine residue that are found to be conserved in all known AgrB-homologues (37, 46). These residues are critical for processing of AgrD by AgrB and GBAP by FsrB and might also serve this function in LamB. During the course of the LC/MS analysis, two other nisin-induced substances

were detected in the mass chromatograms at *m/z* values of 559 (small peak at retention time of 40 min in Fig. 4.5A) and 678 (peak at retention time 29 min), respectively (data not shown). These two compounds may be by-products or precursors in the biosynthesis of LamD558. LamD558 was also detected in the extracted ion chromatograms of the wild-type strain. From the ultraviolet absorption at 280 nm of this peak, the concentration in the mid-log phase culture supernatant was approximately 100 nM, which was several-fold higher than that of staphylococcal autoinducing peptides (30, 49). The LamD558 peak was also detected in culture supernatants of the *lamA* knockout strain. However, it was clearly decreased compared to that from wild-type strain. These results suggest that the expression of the *lamBDCA* operon is positively regulated by the LamCA two-component regulatory system, but a certain level of this expression occurs independently of this regulatory system. The time-course data of LamD558-production in CDM culture supernatants of the wild-type strain showed that production of LamD558 was strongly increased from mid to late-log phase, which coincides with increased *lamBDCA* gene-expression from mid-log phase on, as observed by Northern hybridization analysis (see above). The *lam*-system therefore appears to show growth-phase dependent gene-expression, as expected from the gene annotation as an *agr*-like quorum sensing system.

Finally, induction with the LamD-encoded peptide LamD558 confirmed that LamD558 influenced lp_1204 gene-expression as was observed using microarray analysis. However, it did not indicate an autoregulatory role of LamD558 in *lam* gene-expression. It might be that the LamD558-structure that was determined is not the active autoinducing structure. In addition, it could be that other (competing) peptides or regulatory systems are involved in regulation of *lam* gene-expression. Further experiments are necessary to elucidate the regulatory mechanisms involved.

In conclusion, this is the first complete *agr*-like TCS described in lactobacilli that encodes a cyclic thiolactone AIP. Complete *agrBDCA*-like systems have until now only been detected and described in pathogenic bacteria such as staphylococci (11), *E. faecalis* (36) and *Listeria monocytogenes* (2) that have invasive properties or otherwise interact with host cells (18, 52). In analogy the *L. plantarum lamBDCA*-system might play a role in commensal host-microbe interactions, considering the observed effects on the capacity of a *lamA* mutant to adhere to surfaces (10).

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A regulator containing an AraC-type helix-turn-helix DNA-binding domain is involved in regulation of cell surface properties in *Lactobacillus plantarum*

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Abstract

We have performed the functional characterization of the *aapR* gene of *Lactobacillus plantarum* WCFS1 (lp_3060) coding for a transcriptional regulator that contains an AraC-type helix-turn-helix DNA-binding domain. The *aapR* gene was located downstream of a quorum sensing-like two-component histidine protein kinase gene (*hpk9*) and upstream of the *aapA* gene (lp_3059) encoding a cell-surface protein containing a LPxTG-type cell-wall anchor. Co-transcription of *aapR* and *aapA* was shown by RT-PCR, indicating a functional link between the *aapR* and *aapA* gene products, while *aapR* was not co-transcribed with *hpk9*. An *aapR* deletion-mutant was constructed, which showed no differences in growth-rate and cell- or colony morphology between wild-type and mutant. Analysis of the *aapR* deletion-mutant in an adherence-assay showed that deletion of *aapR* increases the ability of *L. plantarum* to adhere to glass and influences co-aggregation of cells. This effect could be reversed by complementation of the *aapR* gene, indicating that *aapR* controls these phenotypic traits of *L. plantarum*, probably via modulation of cell surface protein expression. In conclusion, these are the first data that point towards the presence of a regulatory gene that is involved in cell surface properties of lactobacilli.

Introduction

Lactic acid bacteria (LAB) play an important role in many food fermentations such as fermented dairy, meat and vegetable products (6, 25), but they are also commonly found as inhabitants of the human gastrointestinal (GI) tract (34). Among the LAB, in particular strains from the genus *Lactobacillus* are implicated to promote intestinal health in man and animals (24). *Lactobacillus plantarum* is a highly adaptive species that is encountered in diverse environmental niches, which include fermented food products and plant material (7, 8), and the human GI tract (1). *L. plantarum* WCFS1 is a single colony isolate from strain NCIMB8826, which was originally isolated from human saliva (14) and was shown to survive stomach passage in an active form and to persist for up to 7 days in the human GI tract after single dosage (35). The 3.3-Mb *L. plantarum* WCFS1 genome is among the largest genomes known in LAB, and encodes a diverse repertoire of extracellular proteins, sugar and amino acid import and utilization proteins, as well as many regulatory genes, reflecting the highly adaptive nature of this microbe (18). Cell-surface proteins are thought to play an important role in colonization of the GI tract by adhesion to enterocytes. Several proteins have been identified in lactobacilli that could be involved in such interactions, such as mucus-binding proteins in *L. reuteri* and *L. fermentum* (29, 30). Genome analysis of the intestinal bacterium *L. johnsonii* identified 117 putative extracellular proteins, of which 42 cell-envelope anchored and secreted proteins (including 14 LPxTG-type cell-wall anchor proteins) that might play a role in host-microbe interactions (4, 28). The *L. plantarum* WCFS1 genome is predicted to encode 211 extracellular proteins, of which nearly 90% are predicted to be cell-envelope anchored proteins (including 25 LPxTG-type cell-wall anchor proteins), which is in agreement with a potential to associate with many different surfaces and substrates in a variety of ecological niches (4, 18, 28). Remarkably, the majority of secreted proteins show little homology between *L. johnsonii* and *L. plantarum*.

Here we describe the *aapR* gene of *L. plantarum* WCFS1 (annotated as lp_3060) encoding a predicted regulator protein, containing a C-terminal AraC-type helix-turn-helix DNA-binding domain (AraC-HTH) (Fig. 5.1). The regulator gene is located downstream of a gene encoding a typical HPK_{10}-subfamily (13) two-component histidine protein kinase *hpk9* (annotated as lp_3063) and upstream of the *aapA* gene (annotated as lp_3059) encoding a cell-surface protein precursor with a

LPxTG-type anchor (5). In between *aapR* and *hpk9* a small ORF (lp_3062) is located with homology to the TfoX_C domain (40). The function of the *aapR* gene was studied by mutational analysis followed by phenotypic characterization and suggested *aapR*-dependent regulation of glass-adherence and cell-aggregation.

Materials and Methods

Bacterial strains and growth media

The bacterial strains and plasmids used in this study and their characteristics are shown in Table 5.1. *Escherichia coli* strain JM109 was cultivated aerobically in Luria-Bertani (LB) or Brain-Heart-Infusion (BHI; Difco) broth at 37°C. *L. plantarum* WCFS1 and its derivatives were cultivated in Man-Rogosa-Sharpe broth (MRS; Difco) at 30°C without agitation. *Lactococcus lactis* MG1363 was grown at 30°C, without agitation in M17 broth (Difco) supplemented with 0.5% glucose (GM17). Solid media contained 1.5% (w/v) agar. Where appropriate, antibiotics were added as follows: erythromycin 150 μg ml^{-1} (*E. coli*) or 5 μg ml^{-1} (*L. plantarum* WCFS1 and *Lactococcus lactis* MG1363).

DNA isolation and construction of integration plasmids

Genomic DNA was isolated from 10 ml overnight cultures of *L. plantarum,* essentially as described (3), with additional disruption by bead-beating in a Biospec bead-beater (3x 90 sec at 5000 rpm) and purification by phenol-chloroform extractions and isopropanol precipitation. PCR was performed using proofreading Platinum *Pfx* DNA Polymerase (Invitrogen) and as a template genomic DNA or plasmid pBACe3.6 (generously provided by J. Catanese) (10). Primer-combinations WCFS-3059-*Nde*I with WCFS-3060-*Eco*RI and WCFS-3060-*Kpn*I with WCFS-3063-*Bam*HI were used to amplify the 5'- and 3'-ends of *aapR* and the regions flanking *aapR* (approximately 1 kb on each side), whereas primer-combinations pBAC-sacB-*Eco*RI and pBAC-sacB-*Kpn*I were used to amplify a locus-tagging-sequence that was cloned in between these two fragments (Table 5.2). PCR products were digested with the appropriate restriction enzymes (Gibco BRL) and cloned into the similarly digested integration-vector pUC19ery (33). Plasmids were transformed into *E. coli* JM109 using an established heatshock procedure (Promega). This resulted in plasmid

pAAPR-K21 containing the complete gene replacement cassette with the mutated transcriptional regulator gene, carrying a 500-bp locus-tagging sequence from pBACe3.6.

Gene replacement of the *L. plantarum* WCFS1 *aapR* gene

L. plantarum WCFS1 was transformed by electroporation, essentially as previously described (16), using electro-competent cells of *L. plantarum* that were grown in MRS supplemented with 1% glycine and prepared in 30% polyethyleneglycol-1450 (PEG-1450). *L. plantarum* cells were transformed with 2 μg of integration plasmid pAAPR-K21, and integrants were selected by plating on MRS-agar supplemented with erythromycin and anaerobic incubation at 30°C for 2-4 days. Single colonies were analyzed by colony PCR with plasmid-chromosome crossover-junction primers WCFS-3059-*Nde*I-junction-1 and SacB-control-2 or SacB-control-3 and WCFS-3063-*Bam*HI-junction-4 (Table 5.2), to confirm single crossover integration up- or downstream of the mutation locus. This resulted in the isolation of a single crossover integrant, designated *L. plantarum* WCFS1::pAAPR-K21, in which integration had occurred downstream of *aapR*. This integrant was propagated for 200 generations in MRS without erythromycin to allow for the second homologous recombination event leading to the anticipated *aapR*::tag replacement mutant. Cultures were plated on MRS-agar and randomly isolated colonies subsequently replica-plated on MRS-agar with and without erythromycin. Erythromycin-sensitive (Em^S) colonies were analyzed by colony PCR with plasmid-chromosome crossover-junction primers and aapR-flanking primers RR-3060-F and RR-3060-R (Table 5.2; see above). The anticipated gene organization of the *aapR*::tag locus in the obtained gene replacement mutant (MSΔ*aapR*) was further confirmed by Southern blot analysis using standard procedures (31).

Construction of complementation strains

The *aapR* gene and its promoter-region (192 bp of upstream sequence with potential regulatory repeats) were amplified with primers WCFS-3060-compl-1-*Pst*I and WCFS-3060-compl-3-*Sma*I using Platinum *Pfx* DNA polymerase (Invitrogen). The obtained amplicon (1050 bp) was digested with *Sma*I and *Pst*I and cloned into the similarly digested low-copy shuttle vector pIL252, using *L. lactis* MG1363 as a cloning host (15, 37). The resulting plasmid, designated pAAPR-C13, was isolated

from *L. lactis* cultures by using a modification of the Qiagen midiprep 100 protocol for *E. coli* (Qiagen), including a pre-lysis treatment with lysozyme (100 mg/ml) and RNAse A (100 μg/ml) in THMS-buffer for 2 h at 37°C, in stead of using the Qiagen P1 midiprep-buffer. The complementation plasmid was then transformed into MSΔ*aapR*, while the empty pIL252 vector was transformed in both MSΔ*aapR* and wild-type strains, to serve as controls. Colony PCR using pIL252 specific and *aapR*-flanking primers was performed to confirm the presence of the plasmids. The resulting strains were designated MSΔ*aapR*-C13, MSΔ*aapR*-pIL252 and MS-pIL252, respectively.

Table 5.1 Bacterial strains and plasmids used in this study and their relevant characteristics

Bacterial strains & plasmids	**Relevant properties** [a]	**Reference(s) or source**
Escherichia coli JM109	cloning host for pUC19ery and its derivatives	Promega
Lactococcus lactis MG1363	cloning host for pIL252 and its derivatives	(12)
Lactobacillus plantarum WCFS1	single colony isolate from human saliva isolate NCIMB8826	(14)
Lactobacillus plantarum WCFS1::pAAPR-K21	*L. plantarum* WCFS1 with single chromosomal integration of pAAPR-K21	This work
Lactobacillus plantarum MSΔ*aapR*	DCO mutant in *L. plantarum* WCFS1 with *aapR* replaced by *aapR*::tag gene replacement cassette	This work
MSΔ*aapR*-C13	MSΔ*aapR* complemented with pAAPR-C13	This work
MS-pIL252	Wild-type carrying pIL252	This work
MSΔ*aapR*-pIL252	MSΔ*aapR* carrying pIL252	This work
pUC19ery	Amp^R, Em^R, 3.8 kb derivative of of pUC19 containing 1.1 kb *Hin*PI fragment of pIL253 carrying the Ery^R gene	(33)
pAAPR-K21	pUC19ery vector carrying lp_3060 gene replacement cassette	This work
pIL252	low-copy-number, broad-host-range plasmid, 4.7 kb, Em^R	(32)
pBACe3.6	Bacterial Artificial Chromosome plasmid	(10)
pAAPR-C13	pIL252-vector carrying *aapR*-complementation construct	This work

[a] Em^R, erythromycin resistant; Amp^R, ampicillin resistant; DCO = double crossover

Table 5.2 PCR primers used in this study

Primer	Sequence (5'-3') *
WCFS-3059-*Nde*I	gccgcttgcatatgCTGTTTGGCATACTG
WCFS-3060-*Eco*RI	aaagaattcAAGAAGGAGACCGGTCAAACG
WCFS-3060-*Kpn*I	taaggtaccTAATAAGATCAGCCCCTTG
WCFS-3063-Bam*HI*	ggaggatccACAGATAAAAGTAGTGC
pBAC-sacB-*Eco*RI	cgtgaattcTACACTGACTATTCCG
pBAC-sacB-*Kpn*I	tcaggtaccGTGTTTGAAGTGATCAGC
WCFS-3059-*Nde*I-junction-1	AGCCACACTACCATCAAACG
WCFS-3063-*Bam*HI-junction-4	TTTGAGTCGCTTGGATATGC
SacB-control-2	CATCGATAAACTGCTGAACG
SacB-control-3	CGATGCTGAGTTAGCGAACG
RR-3060-F	CAAGTACTAATCAGTTCGTG
RR-3060-R	ACAGATTATGGGGACAGAAC
WCFS-3060-compl-1-*Pst*I	atagctgcagTTGAGACATAGTATACCTGC
WCFS-3060-compl-3-*Sma*I	agaacccgggATAACCGCTTTTGTGTATTG

* Capitals indicate nucleotides homologous to genome sequence; lower case bold type characters indicate restriction enzyme recognition sequence; lower case characters indicate non-homologous nucleotides.

Table 5.3 RT-PCR primers used in this study

Primer no.	Primer	Sequence (5'-3')
1	Lp3059F	CCCAACAGCGGTCAACACAGC
2	Lp3059R	TTATTCAATGCCCTTTTCTCAACC
3	Lp3060F	GCGCGGTGCAATCCTGAGTC
4	Lp3060R	GCGCCATTTGCCCCGTGTA
5	Lp3063F	TATGGCAATTGCGGCTATCGT
6	Lp3063R	GTTACCCAAAATCCGCACTACT
7	60-59F	TAATGATCAGCACCCAACCACCAA
8	60-59R	CTAAACGACCGCCGCACTGACTC
9	62-60F	GAGAATTGCGCTTACCCACAGTT
10	62-60R	GGCCCGGTAGAATCGAATAAAAT
11	63-62F	GGATTTTGGGTAACTTGCTGGATA
12	63-62R	CGACTTTGCTACCCGTTGACTTTA

Adherence assays

Phenotypic characterization of adherence and cell-aggregation were initially performed after Merritt et al (22), with strains grown in polystyrene petri-dishes (6 cm diameter) containing glass cover-slides and 5 ml MRS. For quantitative measurements strains were grown in 1 ml MRS or MRS containing erythromycin in 24-well plates (TPP, Switzerland)), with glass cover-slides (14 mm diameter; Menzel-Glazer) on the bottom of the wells. Wells containing medium only were used as blanks and a separate plate was used for optical density measurements. Cells were grown for 24 or 48 h at 30˚C after which medium was removed. Wells were washed twice with PBS to remove loosely attached cells and the remaining adhered cells were air-dried for 10 min. For staining, 0.5 ml crystal violet (0.1% in deionized water) was added and incubated 30 min at 22˚C. Wells were washed three times with deionized water and stained attached cells removed by dissolving in 1 ml 96% ethanol. Absorbance was measured at 595 nm, with 6 replicates for each strain. Experiments were performed in independent triplicates and the statistical significance of differences was calculated using the Student's *t* test (two-tailed, two-sample equal variance). Additionally, biofilm-formation was monitored at distinct time-intervals in a time-course from 0 to 25 h using the same procedure.

Scanning Electron Microscopy

Wild-type and MSΔ*aapR* cultures were grown for 24 or 48 h in 24-wells plates with 8 mm diameter glass slides (Menzel-Glaser) on the bottom, before sample-preparation. Nucleopore Polycarbonate membranes (Costar, Cambridge, USA) with 1 μm pores were incubated for 30 min in a 0.01% poly-L-lysine solution in 0.1M Tris/HCl buffer. Planktonic cells were spotted on poly-L-lysine coated polycarbonate membranes and glass-adhered cells treated while attached to the glass-slides. Bacteria were fixed for 30 min in 3% glutaraldehyde, membranes and glass slides washed 3 times with deionized water, dehydrated with ethanol, using 30%, 50%, 70%, 90% and finally 3x 100% ethanol, and critical-point dried by the CO_2 method (Balzers CPD 020, Balzers Union, Liechtenstein). Dried membranes and glass slides were mounted on sample holders by carbon-adhesive tabs (Electron Microscopy Sciences, Washington, USA). Sample holders were positioned inside a sputter coater (JEOL JFS 1200 Fine Coater) and samples sputter coated with 10 nm gold and analyzed in a

scanning electron microscope (JEOL JSM-5600 LV) at 3 kV. Images were recorded digitally.

RNA extractions

Cultures of wild-type and MSΔ*aapR* were aerobically grown in 100 ml MRS broth at 30ºC starting from an OD_{600} of 0.3. Samples (25 ml) were harvested at early-, mid- and late-log growth-phases (3, 5, 9 h) and immediately quenched and mixed in 4 volumes of quenching-buffer at –80ºC to stop further transcription (60% methanol, 66.7 mM HEPES pH 6.5) after (26). Subsequently, samples were pelleted by centrifugation at -20ºC in a pre-chilled centrifuge and cells resuspended in 0.5 ml cold TE-buffer. RNA was isolated according to the Macaloid-method, essentially as described (19), and further purified by on-column DNaseI treatment on RNeasy columns (Qiagen).

For RNA isolation from glass-adhered cells of wild-type and MSΔ*aapR*, strains were grown overnight as dilution-series in 10 ml MRS at 30ºC. Mid-log cultures were diluted in MRS to an OD_{600} of 0.1, and 10 ml aliquots divided in a 6-well plate (TPP, Switzerland) in triplicate for each time-point (3 replicates for pooling). At 24 h post-inoculation (see time-course) plates were removed and OD_{600} measured. The medium, containing planktonic cells, was pooled from 3 wells and immediately added to centrifuge-tubes containing 4 volumes quenching buffer. Adhered cells were resuspended in the wells in 10 ml MRS, pooled from 3 wells and immediately added to quenching buffer. RNA was further isolated as described above.

Northern blot analysis of *aapR* and flanking genes

RNA was isolated as described above and 10.0 µg RNA sample or 3.0 µl RNA size-marker (Invitrogen) were used for Northern blot analysis by glyoxal denaturation as described (31). RNA was fixed to the membrane by auto-crosslinking in an UV Stratalinker and Northern hybridization performed overnight at 65°C in QuikHyb hybridization buffer (Stratagene) as described (31). Blots were analyzed by exposure to Phosphor screens (Molecular Dynamics) and scanned on a Storm Image Scanner (Molecular Dynamics). DNA probes were made using a Nick Translation kit (Invitrogen).

Reverse transcriptase PCR detection of *aapR* and flanking genes

RT-PCR of *aapR* and flanking genes was performed using the gene-specific primers shown in Table 5.3. cDNA was synthesized using Superscript III (Invitrogen) according to the manufacturer's instructions, using 1.3 μg total RNA isolated from glass-adhered cells as a template (see above). PCR was performed using the following conditions: denaturation 94ºC 5 min; 25 cycles of 94ºC 30 sec, 58ºC 30 sec, 72ºC 30 sec; elongation 72ºC 5 min. A portion of each reaction was analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

Results and Discussion

Transcriptional organization of the *aapR* gene and its flanking region

The genome of *L. plantarum* WCFS1 contains gene lp_3060 that is predicted to code for a regulator protein containing a C-terminal AraC-type HTH DNA-binding domain (11) (Fig. 5.1). In addition to AraC-type DNA-binding domain containing proteins (COG2207), only response regulators containing a CheY-like receiver-domain and AraC-type DNA-binding domain showed significant similarities (COG4753). However, upon closer examination the predicted N-terminal domain of the lp_3060-encoded protein did not contain the characteristic CheY-like receiver-domain as is found in response regulators (RR) of two-component regulatory systems (TCS).

Most AraC/XylS family proteins are small with an average size of 250-300 residues and are transcriptional activators, although in some cases they can act as repressors (CelD in *Escherichia coli*) or have dual functions (AraC from *E. coli* and YbtA from *Yersinia pestis*) (11). AraC/XylS-type transcriptional regulators in general appear to be involved in carbon metabolism, stress response or pathogenesis, and often regulate production of cell surface proteins, such as adhesins, fimbriae or cell capsules. Gene lp_3060 is predicted to code for a 253-residue protein with a clear AraC-type HTH domain (SMART database entry smart00342) and is located upstream of gene lp_3059, which is predicted to encode a large 1356-residue cell-surface protein containing a LPxTG-type cell-wall anchor and showing partial homology to repeat-domains of the mucus-binding protein precursor (Mub) of *Lactobacillus reuterii* (30) (Fig. 5.1A). Considering the above, lp_3060 is likely to

code for a transcriptional regulator that might be directly activated or repressed by binding of small ligands (e.g. sugars) or larger proteins, as reported previously (11, 27). In addition, the genetic organization of lp_3060 with lp_3059 suggested a functional link between these genes and a role in cell-surface or adherence related properties (see also below). Hence, this putative operon was designated *aap* for adherence-associated proteins, consisting of *aapR* (lp_3060), the transcriptional regulator of adherence associated proteins, and *aapA* (lp_3059), the adherence-associated cell-surface protein.

The *aapR* gene is also located downstream of a two-component histidine protein kinase gene *hpk9* (lp_3063). The predicted HPK9 protein is a typical representative of peptide-based quorum sensing-like HPKs as it contains HPK_{10}-subfamily motifs (13), as well as six predicted trans-membrane-segments (TMS) (Fig. 5.1). Quorum sensing (QS) two-component regulatory systems show a comparable paired organization of HPK and regulator, and this may suggest that *aapR* and *hpk9* actually constitute such a system. In addition, in between *aapR* and *hpk9* a small ORF is located (lp_3062) that is predicted to encode a 94-residue protein with homology to the C-terminal domain of the TfoX protein of *Haemophilus influenzae* (Pfam accession number PF04994). The latter TfoX protein has been suggested to play a key role in cell density-dependent regulation of genetic competence, and the C-terminal domain might function autonomously (17, 40).

Taken together, the detailed annotation of *aapR*, lp_3062 and *hpk9* suggested that they might form an unusual signal transduction module that could be involved in cell density-dependent gene regulation. In addition, *aapA* and *aapR* could be involved in regulating cell-surface properties. For this reason, Northern hybridization analysis was performed at different growth phases for cultures grown in MRS broth, to elucidate if these genes were genetically linked in an operon structure and showed cell-density-dependent expression. Using probes targeting *aapR* and *hpk9*, heterogeneously sized transcripts were detected that increased in intensity at later stages of growth (Fig. 5.2). However, no clear bands could be detected and rather a smear was found that was partly suppressed at the location of the abundant 16S and 23S rRNA. The upper size of the transcripts was difficult to assess, but was estimated to be approximately 5.0 kb for the *aapR*-specific products and 4.3 kb for the *hpk9*-specific products (Fig. 5.2). While both transcripts showed a large size, they appeared to differ because of a slightly different kinetics of production, size and extent of

degradation. Hybridization of the same blots with an *aapA*-targeting probe, showed that a 5.0-kb transcript hybridized with this probe. This indicated that the 0.8-kb *aapR* gene and 4.1-kb *aapA* gene were co-transcribed (Fig. 5.2). The predicted transcriptional organization is compatible with the presence of obvious stem-loop structures that could function as transcriptional termination signals (Fig. 5.1A). To confirm the transcriptional linkage between the *aapR* and *aapA* genes, we performed RT-PCR on mRNA isolated from late-exponential phase cultures. In this way we could show that *aapR* and *aapA* are indeed co-transcribed, while *hpk9* and lp_3062 are transcribed separately as another transcript (see Fig. 5.1B). Remarkably, the *hpk9*-lp_3062-*aapR*-*aapA* gene cluster is located in a so-called lifestyle-adaptation region, which contains genes that most probably have been acquired recently by horizontal gene transfer, based on the deviating base composition and functional overrepresentation in this region (23).

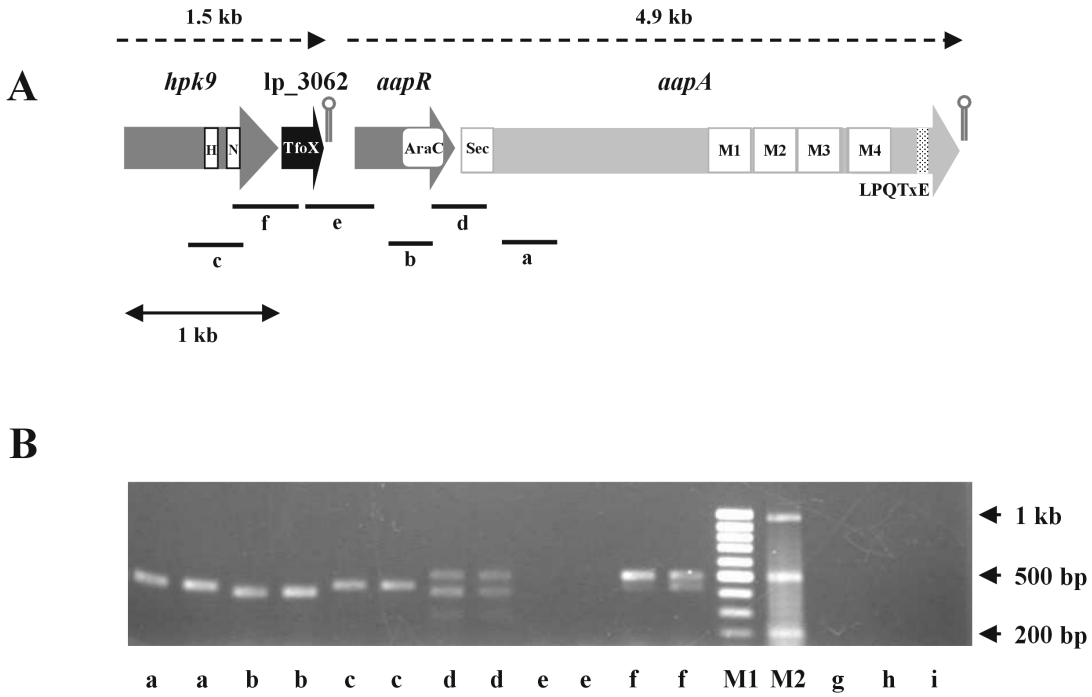

Figure 5.1 (A) Genetic organization of *aapR* (lp_3060) and its flanking regions, as deduced from the *L. plantarum* WCFS1 genome project (http://www.cmbi.kun.nl/lactobacillus). Stemloops indicate putative terminator-structures (free energies of ΔG = -19.2 and –20.3 kcal/mol respectively), as determined by using the RNA-folding program Mfold. *hpk9* (lp_3063): histidine protein kinase. H and N: characteristic HDY and NAE-boxes of HPK_{10}-subfamily (13). TfoX_C: homologous to TfoX C-terminal domain of *Haemophilus influenzae* (Pfam database entry pfam04994). AraC: AraC-HTH domain (AraC/XylS domain signatures can be accessed as PROSITE database entries PS01124 and PS00041). *aapA* (lp_3059): cell-surface protein containing a LPxTG-type motif for processing by sortase (LPQTxE). Sec: signal sequence for Sec-dependent secretion. M1-4: Mub-type repeats. Black bars below the genes (a-f) represent fragments amplified by RT-PCR and dashed lines indicate the deduced transcriptional organization of *aapR* and its flanking genes. (B) Results of RT-PCR experiments of *aapR* and flanking genes. RT-PCR products were electrophoresed on a 1% agarose gel. Lanes a: primers 1-2; lanes b: primers 3-4; lanes c: primers 5-6; lanes d: primers 7-8; lanes e: primers 9-10; lanes f: primers 11-12; lane g: primers 7-8 without RT; lane h: primers 9-10 without RT; lane i: primers 11-12 without RT. M1: 100 bp DNA size marker; M2: DNA size marker. Primer numbers are described in Table 5.3 and a-f correspond to the RT-PCR amplified fragments depicted in (A).

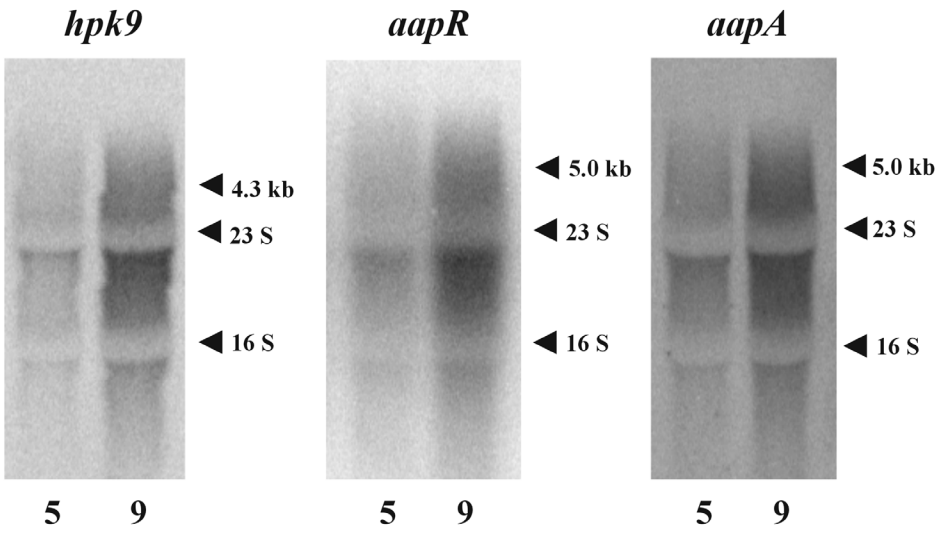

Figure 5.2 Northern blot analysis of *hpk9*, *aapR* and *aapA* expression after 5 and 9 h growth of *L. plantarum* wild-type in MRS broth. Similar amounts of RNA (10 µg) were loaded in all lanes.

Adherence characteristics of wild-type and *aapR* deletion-mutant

To characterize the function of the *aapR* gene, a gene-replacement mutant was constructed (MSΔ*aapR*) and confirmed by PCR and Southern hybridization (data not shown). Initial experiments showed no differences in growth-rate and cell- or colony morphology between the wild-type and *aapR* mutant strain (data not shown). In many Gram-positive bacteria quorum sensing TCS have been shown to be involved in biofilm formation (20, 36, 39). In addition, AraC/XylS-type transcriptional regulators in many cases are involved in production of cell surface proteins (11), and in *Staphylococcus aureus* the *rbf* gene, which contains an AraC/XylS consensus signature, acts as an activator in NaCl- and glucose-induced biofilm formation (21). Since the *aapR*-encoded protein contains an AraC-domain too and appeared to be co-transcribed with the LPxTG-type cell-wall protein-encoding gene *aapA*, we decided to investigate the adherence properties of wild-type and *aapR*-mutant cultures. Initial experiments were performed in polystyrene petri-dishes for cultures grown in MRS or CDM, with or without glass cover-slides, as described by Merritt et al (2003). The *aapR* mutant clearly showed more and larger cell-aggregates after 24 h of growth, compared to the wild-type, and appeared to adhere more to the glass-slide in MRS (Fig. 5.3A and B). This aggregation-phenotype was not observed in a Chemically Defined Medium (CDM) and required the presence of a glass-surface, indicating that a hydrophilic surface might be necessary for cell-aggregation and adherence. To

quantify the number of adhered cells, cultures were grown in MRS in 24-well plates containing glass slides and glass-adhered cells quantified after staining with crystal violet. This assay confirmed that the mutant had increased glass-adherence, which became more prominent after longer incubation times (Fig. 5.3C). The cell-aggregation that was observed for the *aapR*-mutant when grown in petri-dishes, was not observed using the 24-well plate-assay. To further confirm the role of *aapR* in the adherence phenotype, the mutant strain was complemented with gene *aapR* cloned in the low-copy shuttle vector pIL252. Adherence of the complemented mutants was almost restored to wild-type levels, indicating that *aapR* is indeed involved in the adherence-phenotype (Figs. 5.3B and C). The observed differences were all significant according to the Student's *t* test ($P<0.001$).

The increased adherence after longer incubation times prompted us to analyze the time-course of adherence. This showed that the differences between wild-type and MSΔ*aapR* became manifest after approximately 10 h of growth (Fig. 5.4). This indicated a cell density-dependent regulation of the adherence phenotype, with an onset in stationary phase, and it is likely that the *aapR-aapA* gene cluster is involved in this phenomenon. Scanning electron microscopy (SEM) of glass-adhered cells was performed as differences in biofilm-structures might be expected, based on the glass-adherence properties. This revealed for both wild-type and mutant a densely packed biofilm of glass-adhered cells, without pronounced differences, while the cell morphology at larger magnifications showed the same smooth appearance for both strains (data not shown). The strong adherence to glass allowed for the separate recovery of biofilm and planktonic cells, and total RNA was isolated from both cell populations. Northern hybridization analysis of RNA isolated from these cells showed significantly lower expression of *aapA* in planktonic cells than in the glass-adhered cells (Fig. 5.5A), and indicated the presence of heterogeneously sized transcripts in the range of 3 to 6 kb. These transcripts were also observed with an *aapR*-targeted probe, confirming the co-transcription of *aapA* and *aapR*, as was found by RT-PCR (see above). Moreover, the *aapR*-expression was clearly decreased, while the *aapA*-expression was decreased to a lesser extent, in the *aapR*-mutant compared to the wild-type (Fig. 5.5A and B).

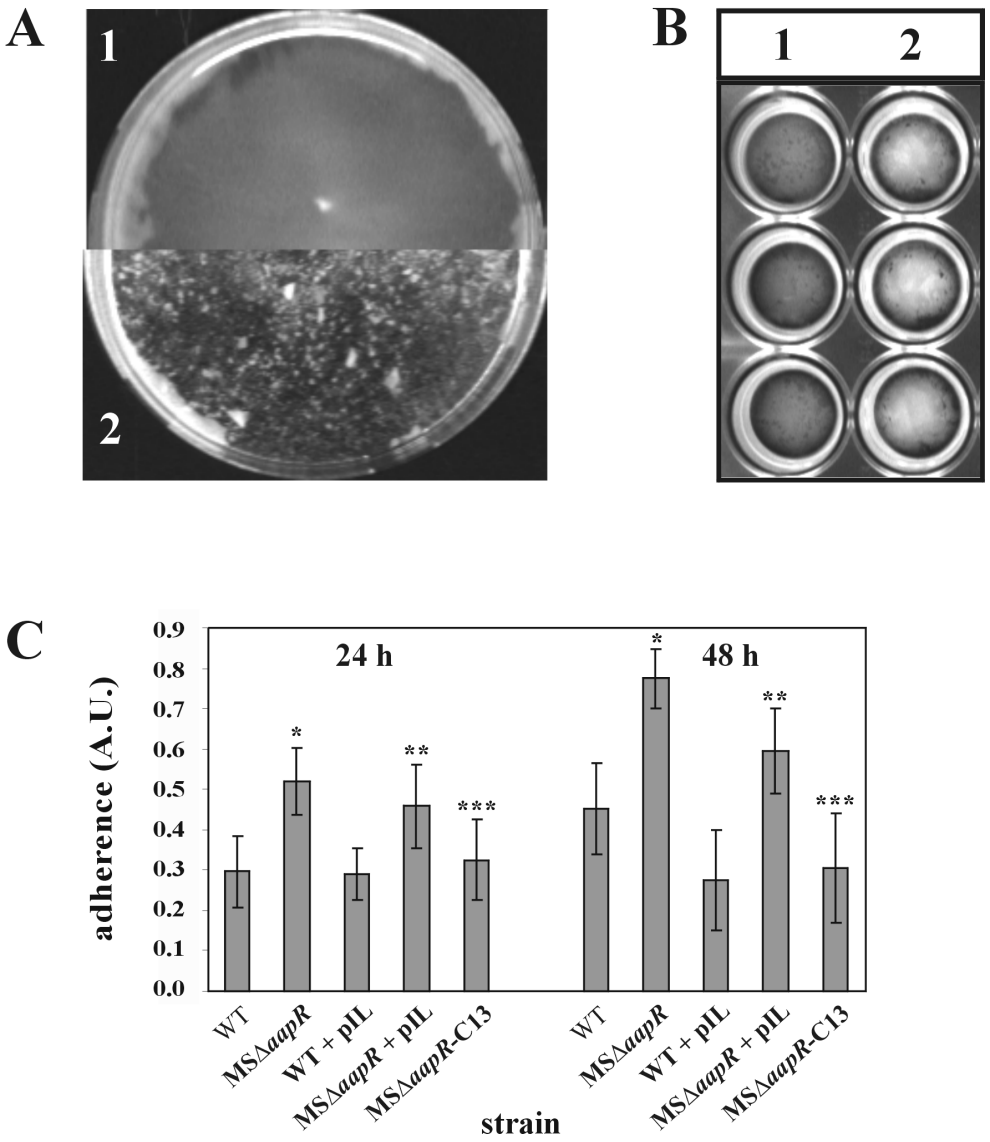

Figure 5.3 Aggregation and adherence phenotypes of wild-type, *aapR*-mutant and *aapR*-complemented strains. (A) Petri-dish assay showing cell-aggregation in MRS with glass cover slides for wild-type (1) and MSΔ*aapR* (2) strains after 24 h of growth at 30°C. (B) Photographs of glass-adhered cells grown in 24-well plates in MRS with glass cover slides after the phosphate-buffered saline wash-step for wild-type (1) and MSΔ*aapR* (2) strains after 48 h of growth at 30°C. Three replicates are shown. (C) Quantification of crystal violet-stained glass-adhered cells in 24-well plate assay after 24 and 48 h of growth at 30°C. Adherence was measured in arbitrary units (A.U.). Means ± standard errors (error bars) of three 24-well plates, with six wells measured per plate are shown. Wild-type (WT) and *aapR*-mutant (MSΔ*aapR*) strains were grown in MRS. WT+pIL and MSΔ*aapR*+pIL: wild-type and *aapR*-mutant strain with empty vector pIL252, and MSΔ*aapR*-C13: *aapR*-mutant strain complemented with pAAPR-C13, were all grown in MRS containing erythromycine. Single, double and triple asterisks denote statistically significant different values (P<0.001) of MSΔ*aapR* versus wild-type, MSΔ*aapR*+pIL versus wild-type+pIL, or MSΔ*aapR*-C13 versus MSΔ*aapR*+pIL, respectively. Statistical comparisons were done using the Student's *t* test (two-tailed, two-sample equal variance).

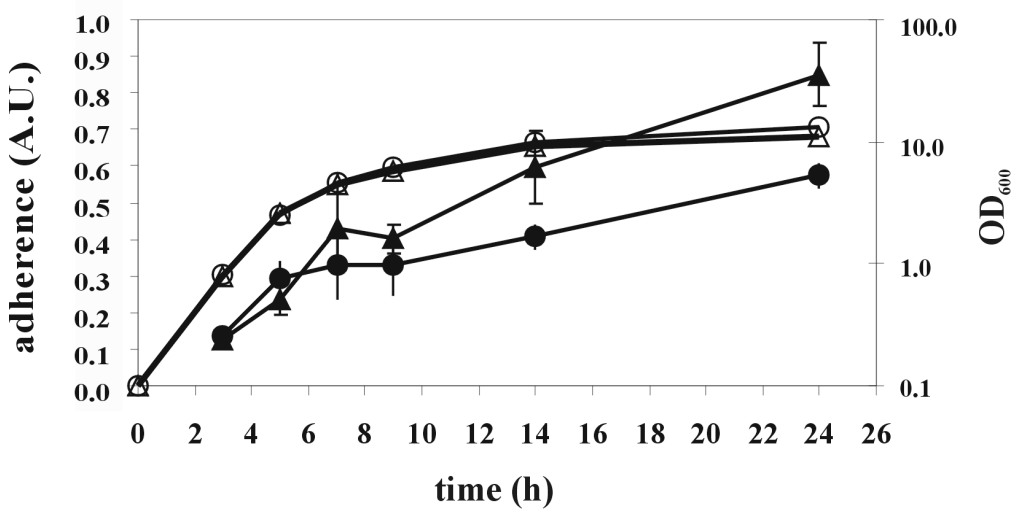

Figure 5.4 Timecourse of glass-adherence. Open circles: wild-type OD_{600}; closed circles: wild-type adherence; open triangles: MSΔ*aapR* OD_{600}; closed triangles: MSΔ*aapR* adherence.

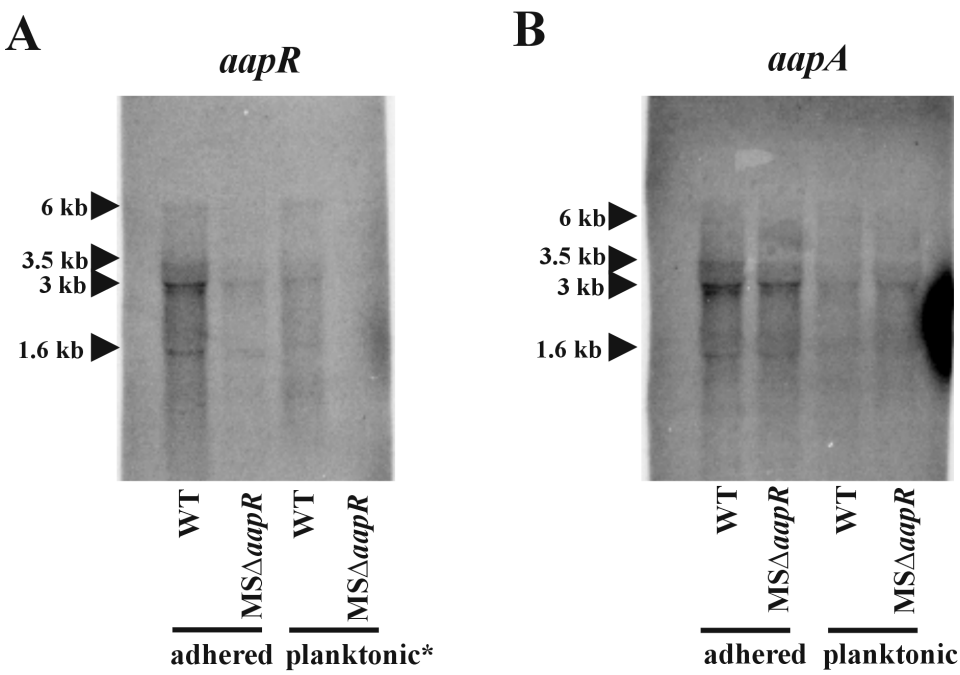

Figure 5.5 Northern blot analysis of (A) *aapR*-expression in adhered and planktonic cells of *L. plantarum* wild-type and MSΔ*aapR* grown in MRS broth, and (B) *aapA*-expression in adhered and planktonic cells of *L. plantarum* wild-type and MSΔ*aapR*. In all lanes 10 µg of total RNA was loaded, except for planktonic* 5 µg was loaded.

Functional role of *aapR* and *aapA* and their paralogues

The *L. plantarum* genome was predicted to encode three additional genes that contained Mub-like domains and showed partial homology (20-27% protein identity) to *aapA*: genes lp_1229, lp_1643, and lp_3114. Proteins containing these Mub-like domains have only been detected in lactobacilli and pediococci (J. Boekhorst, personal communication), and their function in adhesion has been experimentally demonstrated for the Mub protein of *L. reuterii* (30) and lp_1229 in *L. plantarum* (G. Pretzer, personal communication). While the Mub protein of *L. reuterii* is very large (3269 aa) and contains fourteen copies of a Mub repeat, the homologues in *L. plantarum* are considerably smaller (1010 to 2219 aa) and contain only 3 to 6 Mub repeats. Also regulator gene *aapR* has two paralogues genes: lp_1227a + lp_1228 (split because of frameshift) and lp_3415, showing 22 and 25% protein identity, respectively (J. Boekhorst, personal communication). The homology is mainly in the AraC-type C-terminus, while the N-termini are more different. Surprisingly, regulator lp_1227a-1228 is located upstream of the extracellular adherence protein lp_1229 (see above), and regulator lp_3415 downstream of the extracellular proteins lp_3412-3414, albeit both divergent, which resembles the organization for *aapR* and *aapA*. It is tempting to speculate, considering the present experimental data, that these 3 AraC-type regulators are involved in regulating the production of the adjacent extracellular proteins. Preliminary clone-based micro-array analysis of global gene expression in the *aapR*-mutant versus wild-type indicates that a diverse set of genes is affected by the mutation. Remarkably, among these were many genes encoding cell-surface and extracellular proteins, suggesting that the observed adherence effects do not only involve *aapR* and *aapA* (unpublished observations).

Biofilm formation of *L. plantarum* strains has not been studied well, with the only data available those on *L. plantarum* biofilm growth promoted by *Actinomyces* species and *Streptococcus mutans* (9). These studies were performed on Thermanox™ coverslips, a plastic material with a hydrophilic surface-treated assay side, in a chemically defined saliva-analog medium (38). Biofilm formation in our experiments was observed on a hydrophilic glass-surface too, while no clear biofilms were formed on hydrophobic polystyrene surfaces (data not shown), with cells grown in MRS.

To our knowledge, these data are the first that point towards the presence of a regulatory gene that is involved in adherence properties of lactobacilli. Homologues of the *aapR* gene appear to be present in other *L. plantarum* strains (23), but not in *L.*

johnsonii (4) or *L. acidophilus* (2), suggesting a species-specific function of this gene. The *aapR* gene shows homology to response regulators containing a N-terminal CheY-like receiver-domain and a C-terminal AraC-type HTH DNA-binding domain (COG4753). The function of these response regulators has not been determined yet, and it remains to be seen if they constitute TCS together with a HPK.

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Summary and concluding remarks

This thesis describes the functional analysis of quorum sensing regulatory systems in the humane isolate *Lactobacillus plantarum* WCFS1. It shows the potential of genomics and molecular tools for elucidating the functional roles of uncharacterized regulatory genes in the annotated *L. plantarum* genome. The most important results are summarized below and will be briefly discussed. In addition, the implications of these results on future quorum sensing research in lactobacilli and their use in public health related issues are mentioned.

Two-component regulatory systems and cell-to-cell communication in *L. plantarum*

In Chapter 1 general features of *L. plantarum* WCFS1 and its 3.3 Mb genome (29) are described and a brief outline of this thesis is presented, while Chapter 2 gives an overview on peptide-based quorum sensing in Gram-positive bacteria. The *L. plantarum* genome was predicted to encode a large number (261) of regulatory genes that are thought to be a reflection of the many different environmental conditions that *L. plantarum* faces. Among these were 26 two-component histidine protein kinase (HPK) and response regulator (RR) genes that constituted 13 genetically linked, complete two-component signal transduction systems (TCS), as well as one orphan HPK and one orphan RR on different positions on the genome that might constitute a fourteenth TCS. These TCS were anticipated to be involved in the detection of (specific) environmental signals, but also in the response to specific secreted signaling molecules in quorum sensing (56). In Chapter 3 the genomics analysis of these (quorum sensing) TCS in *L. plantarum* is described. Four of the TCS showed significant similarities to those involved in peptide-based quorum sensing, and these were analyzed using bioinformatics and functional genomic approaches. Three TCS contained an upstream gene encoding a putative autoinducing peptide (AIP). Two of the latter TCS were preceded by an AIP with a double-glycine-type leader-peptide, and one of these was identical to the *plnABCD* regulatory system that regulates plantaricin production.

A third TCS that was described in Chapter 4, showed a similar genetic organization as the *Staphylococcus aureus agrBDCA* quorum sensing system, and was designated *lamBDCA* for *Lactobacillus agr*-like module. Functional analysis of the *lam*-system showed its involvement in biofilm formation and regulation of genes

encoding cell surface proteins. The *lamD* gene of this system was predicted to encode a cyclic thiolactone pentapeptide, which is a novel type of signalling molecule in lactobacilli. A fourth TCS was paralogous to the *lam*-system, but lacked the *lamBD* genes. Finally, an orphan HPK and RR showed clear peptide-based quorum sensing characteristics, and could therefore form a fifth peptide-based quorum sensing TCS. Besides these peptide-based quorum sensing TCS, the *luxS* gene encoding the autoinducer-2 synthase was identified and analysis showed the production of an autoinducer-2 like molecule.

In Chapter 5 the functional analysis was described of regulator gene *aapR*, containing an AraC-type HTH DNA-binding domain, and showed its involvement in biofilm formation and cell-aggregation.

The most extensively studied peptide-based quorum sensing TCS in lactic acid bacteria (LAB) up to date, are those involved in the production of antimicrobial peptides, such as lantibiotics (class I antimicrobial peptides) (34) or nonlantibiotics (class II antimicrobial peptides) (28, 40). The AIPs activating the TCS involved in class II antimicrobial peptide production in general are non-modified linear peptides that contain a typical double-glycine-type leader peptide, which is cleaved from the precursor peptide during export by a dedicated ABC-transporter with a Peptidase C39 protease-family domain (22). This simple quorum sensing system is widely spread and has been well described in many LAB including lactobacilli, carnobacteria, and enterococci (28, 40) and two TCS encoding such an AIP were also present on the *L. plantarum* WCFS1 genome. Lantibiotics are post-translationally modified AIPs that may serve both a signaling and antimicrobial function in LAB. The modified precursor peptides are exported by a dedicated ABC exporter and activated by proteolytic removal of a leader sequence, which in several cases is catalyzed by a specific leader peptidase (34). TCS that could encode for lantibiotics were not detected on the *L. plantarum* WCFS1 genome. Homologues of the peptide-based *agr* quorum sensing systems of staphylococci (41) have not been described in LAB, with the exception of the *fsr*-system in *Enterococcus faecalis* (49). This thesis now provides the first example of a complete *agr*-system in the genus *Lactobacillus*, and indicates that *agr*-like and possibly other novel types of quorum sensing systems might be more widespread among other species and genera of the LAB group, then was thought before. Finally, autoinducer-2 (AI-2), which is a furanosyl-borate-diester,

has been detected in both Gram-negative and Gram-positive bacteria, including LAB (10). It may therefore serve as an interspecies signaling molecule (62), and be involved in microbial interactions between intestinal bacteria.

Cell-to-cell communication in natural ecosystems of LAB

The high cell densities and varying conditions of environments in which LAB are found - such as the GI-tract and industrial and food settings - favour the development of quorum sensing systems that increases their competitive and adaptive fitness or improves interactions with the host. To ensure specificity of the quorum sensing response in these complex ecosystems, it is expected that the quorum sensing regulatory systems show sufficient genetic diversity. Diversity and specificity of quorum sensing systems in related species and even strains, has been well studied for the *agr*-system in *S. aureus* strains (29), and the competence-systems in *B. subtilis* (60) and *Streptococcus* spp. (61). These studies showed the existence of strain-specific peptide pheromone types (pherotypes), and demonstrated intra- and interspecies cross-talk (both cross-inhibition and cross-induction). Cross-talk by AIPs between different strains and species could play an important role in the way microbial ecosystems develop and function, while cross-inhibition of other bacteria is expected to be advantageous during colonisation of abiotic or biotic surfaces or competition for substrates in e.g. dairy products. For LAB, studies on AIP diversity and specificity have not been performed yet, but they could be valuable in understanding the microbial processes in their natural habitats. Until now, most studies in LAB have focused on quorum sensing regulation of bacteriocin production and their role in microbial competition (14, 34). Production of these natural inhibitors is used to control growth of competing micro-organisms, thereby giving an advantage to the bacteriocin-producing species in colonisation of ecological niches or access to nutrients. One can imagine that inhibition or induction by AIP cross-talk also might serve such a role. In addition, quorum sensing cross-talk could also lead to co-ordinated co-metabolic conversions by bacterial species or interference with host responses. Indications for quorum sensing-related functional co-ordination between commensal bacteria and their host come from studies on the production of the lantibiotic ruminococcin A (RumA) in the human commensal *Ruminococcus gnavus* FRE1 (19). RumA production was shown to be dependent on the presence of trypsin,

which was suggested to be involved in processing of a putative extracellular AIP that might act as the inducing signal for the regulatory TCS involved in RumA production. Such activation could be a specific adaptation to the intestinal environment, in which this strain naturally resides, and might be a mechanism that is also used by other intestinal bacteria. Finally, the quorum sensing system involved in expression of virulence genes in enterohemorrhagic *E. coli* (EHEC) could be activated by the bacterial autoinducer-3 (AI-3), whose synthesis depends on *luxS*, but also by the human hormone epinephrine. This indicates a novel mechanism of host-microbe inter-kingdom cross-talk (54), which might be more widespread considering the presence of *luxS*-homologues in a large variety of bacteria.

Biofilm formation in bacteria (24, 55) has been thought to be ecologically advantageous, by providing protection against dehydration or antibiotics, increasing nutrient availability and metabolic co-operativity, or enhancing gene transfer. Biofilm formation in Gram-positive bacteria has mainly been studied for pathogens such as staphylococci that grow on polymeric medical devices (20), and streptococci that are involved in infections (6) or can be found in complex microbial ecosystems such as dental plaques in the oral cavity (31). However, biofilms of commensal bacteria were recently also detected in the gut mucus lining of the gastrointestinal tract (45). Biofilm formation in many cases involves peptide-based quorum sensing mechanisms (46), and studies have indicated that biofilm formation, in staphylococci at least, requires both environmental as well as quorum sensing signals. For *L. plantarum* a recent report showed that *in vitro* biofilm formation of *L. plantarum* is promoted in co-culture (16), while this thesis demonstrates the involvement of regulatory genes related to *agr*-like systems (57) and AraC-type regulators in this process. Whether *L. plantarum* actually forms biofilms in its natural habitats remains unknown, but is considered to be likely in view of the *in vitro* studies.

Future developments in quorum sensing research on LAB

Many of the genes on the completed *L. plantarum* WCFS1 genome sequence were annotated as such based on comparative genomics, but in most cases no experimental confirmation for the suggested physiological role was available. The completed genomes of many LAB will soon be available (26), and concomitant with that bioinformatics-tools for function-prediction of (regulatory) genes in LAB are

increasingly improving (52). Despite this, genetic and biochemical approaches still will be necessary to confirm the annotations or to assign functions to regulatory genes of unknown architecture. The availability of efficient genetic tools for LAB (18, 35), as well as the development of genomics-technologies (such as microarrays) and proteomics-tools (3, 17) in LAB-research, can aid in this process. Directed genetic approaches to identify the function of known (quorum sensing) two-component regulatory systems have already been used successfully in Gram-negative bacteria such as *Escherichia coli* (63), and Gram-positive bacteria such as *Enterococcus faecalis*, *Bacillus subtilis* and *Streptococcus pneumoniae* (21, 30, 32, 59). In addition, in particular microarrays have been proven successful in identifying novel (quorum sensing) TCS in *S. pneumoniae* (8) and *B. subtilis* (30) or elucidating the global regulatory functions of known quorum sensing TCS such as ComPA in *B. subtilis* (43), ComDE in *S. pneumoniae* (47, 48) or AgrCA in *Staphylococcus aureus* (13). It is expected that the combined use of genome data mining, functional and comparative genomics will also give more insight into metabolic and global regulatory networks (including quorum sensing) in LAB.

In contrast to the high number of studies describing the *in vitro* functionality of quorum sensing systems discovered in LAB, little research has been done on the presence and functionality of these systems in natural microbial ecosystems. Indications of a widespread distribution of quorum sensing in ecosystems come from the discovery of e.g. *N*-acyl homoserine lactones and autoinducer-2 activity in the rumen (15, 36), but also (AIP-controlled) class IIa bacteriocin production in human gastrointestinal LAB such as lactobacilli and enterococci (40) and ruminal bacteria like *Butyrivibrio fibrisolvens* (25). By applying novel genomics and genetics-tools we can now also further address the *in situ* presence, activity and function of quorum sensing systems of LAB in their natural ecosystems, such as the GI-tract or dairy products (9, 37, 39, 42).

The advanced knowledge of the regulation and functions of peptide-based quorum sensing systems may also lead to the development of new tools in molecular biology and applications in medicine. Quorum sensing-regulated systems could be useful for designing controlled gene-expression systems in Gram-positive bacteria, as can be illustrated by the food-grade nisin-controlled expression system (NICE), which is based on the autoregulatory nisin gene cluster from *Lactococcus lactis* (7) or inducible expression-systems based on the sakacin system from *Lactobacillus sakei*

(53). Successful implementation of the NICE-system was demonstrated in various LAB and other low-GC Gram-positive bacteria (2, 27). Knowledge of quorum sensing mechanisms involved in host-microbe interactions could lead to new medical therapies against common pathogens that show increased resistance against antibiotics. Promising developments in this area are largely based on insights into the effects of peptide-structure on the activity of AIPs, which might lead to the rational design of synthetic peptides that are able to inhibit quorum sensing-regulated virulence phenotypes (44). The potential of such a strategy has already been shown in studies on quorum sensing-blocking synthetic peptides that were designed based on knowledge of *S. aureus*-AIP structure-function relationships (33, 51). In other studies concerning targeted inhibition of virulence and infections caused by *S. aureus*, a chimeric peptide of an AgrD-peptide fused to a bacteriocin was shown to specifically kill *S. aureus* cells, but not the related *Staphyloccus epidermidis* or unrelated *Streptococcus pneumoniae* (50), while a chimeric peptide of a RNA III-inhibiting peptide fused to a bacteriocin was shown to prevent staphylococcal infections (1, 11). Similar approaches that target quorum sensing signals might also be applied to the *fsr* and cytolysin systems involved in the production of virulence factors and interaction with host cells, respectively, of *Enterococcus faecalis* another main cause of hospital infections for which few therapeutics exist (4, 5, 38). In analogy, knowledge of the quorum sensing systems of commensal bacteria (including LAB) that are involved in microbe-microbe and host-microbe interactions, might lead to improved understanding of gut functionality, disease prevention, and design of products containing LAB with specific health-promoting properties (58).

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Samenvatting

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Het is al een decennium bekend dat bacteriën interactie met elkaar kunnen hebben door middel van speciale communicatiesystemen. Deze cel-cel communicatie wordt gebruikt om expressie van genen te reguleren die betrokken zijn bij diverse functies, zoals aanhechting aan oppervlakken, virulentie, opname van (vreemd) genetisch materiaal, of de productie van anti-microbiële verbindingen. Activatie van deze genen gebeurt op een gecoördineerde wijze en is afhankelijk van hoge celdichtheden en werd daarom quorum sensing genoemd (quorum betekent hoeveelheid). Door gebruik te maken van speciale uitgescheiden signaalstoffen (feromonen) die zich ophopen bij hoge celdichtheden, kunnen bacteriën deze celdichtheid indirect waarnemen en vervolgens daar op passende wijze op reageren met behulp van speciale genetische regelsystemen. De signaalstoffen die bacteriën uit de groep der Gram-positieve bacteriën gebruiken zijn meestal peptiden (kleine eiwitten). Quorum sensing systemen worden verondersteld te ontstaan in ecosystemen met hoge celdichtheden, waar veel microbiële interacties plaatsvinden en er weinig diffusie is. Deze regelsystemen zouden bacterie-populaties helpen om gecoördineerde reacties te ontwikkelen die tot competitievoordelen of efficiënte aanpassingen op veranderende milieucondities kunnen leiden, of coördinatie van interacties tussen bacteriën en hun biologische omgeving mogelijk maken.

Het menselijke maagdarmkanaal bestaat uit verschillende delen, waarin sterk verschillende omstandigheden voorkomen wat betreft o.a. zuurgraad en aanwezigheid van voedingsstoffen, en herbergt een complex microbieel ecosysteem. De microbiële darmgemeenschap (microbiota) verandert in samenhang met deze omstandigheden en bereikt vaak zeer hoge celdichtheden en een hoge diversiteit (bijvoorbeeld meer dan 1000 verschillende bacteriesoorten in de dikke darm). Interacties tussen microben en de gastheer en microben onderling hebben ook invloed op de samenstelling. Melkzuurbacteriën zijn Gram-positieve bacteriën die algemeen deel uitmaken van de menselijke maagdarm-microbiota en tevens veelvuldig gebruikt worden in voedselfermentaties van bijvoorbeeld zuivel, vlees en plantaardige grondstoffen. Melkzuurbacteriën in het maagdarmkanaal ondervinden vaak grote veranderingen in milieucondities en bewonen dichtbevolkte darmsegmenten, zoals de dunne en dikke darm, terwijl melkzuurbacteriën in de voedselrijke omgeving in industrie en voedingsmiddelen tot hoge dichtheden kunnen groeien. De karakteristieken van deze natuurlijke milieus worden daarom verondersteld gunstig te zijn voor de ontwikkeling

en aanwezigheid van genetische regulatiesystemen in melkzuurbacteriën die specifiek reageren op hoge celdichtheden (quorum sensing). Karakteristieke maagdarm-melkzuurbacteriën zijn o.a. lactobacillen, waarvan sommige soorten gezondheids-bevorderende eigenschappen worden toegedicht. *Lactobacillus plantarum* is een voorbeeld van een flexibele en veelzijdige lactobacillen-soort die men aantreft in diverse ecosystemen zoals gefermenteerd voedsel of op plantenmateriaal. Daarnaast is *L. plantarum* een natuurlijke bewoner van het menselijke maagdarmkanaal en sommige *L. plantarum* stammen worden verkocht als probiotica (met name in zuivelproducten).

De genetische code (genoom) van *L. plantarum* stam WCFS1 is recentelijk opgehelderd en bleek te coderen voor 261 regulatiegenen (8.5% van totaal aantal gecodeerde eiwitten). Dit relatief hoge aantal regulatiegenen wordt verder alleen aangetroffen in andere veelzijdige, flexibele bacteriën zoals de ziekteverwekkende bacteriën *Pseudomonas aeroguinosa* en *Listeria monocytogenes*, en is daarom waarschijnlijk een afspiegeling van de vele verschillende milieuomstandigheden waaraan deze bacteriën vaak blootstaan. In vergelijking, de genomen van de verwante lactobacillen *L. johnsonii* en *L. acidophilus* coderen beiden voor slechts 114 potentiële regulatiegenen (beiden 6% van totale eiwitten), wat een afspiegeling is van het meer stabiele, voedselrijke maagdarm-milieu waar deze soorten voorkomen. Een deel van de regulatiegenen zijn zogenaamde twee-component regulatiesystemen (TCS). Dit zijn genetische regulatiesystemen die uitwendige milieusignalen waarnemen en verwerken, zoals nutriënten (bijv. nitraat, fosfaat of CO_2) en suikers, maar ook fysisch-chemische veranderingen zoals zoutgehalte, temperatuur of zuurgraad waarnemen. Daarnaast worden TCS, met name in Gram-positieve bacteriën, ook gebruikt om te reageren op speciale signaalmoleculen die bacteriën produceren voor quorum sensing regulatie.

Het onderzoek beschreven in dit proefschrift heeft zich gericht op de moleculair biologische analyse van genen die gecodeerd waren op het genoom van de bacterie *L. plantarum* WCFS1 en beschreven waren als quorum sensing regulatiesystemen. Een combinatie van genoomanalyse, moleculair biologische methoden en bioinformatica (m.b.v. computerprogramma's biologische voorspellingen doen) werdt toegepast om voorspellingen te doen over de mogelijke functies van deze genen en dit experimenteel aan te tonen.

In Hoofdstuk 1 werden de algemene eigenschappen van *L. plantarum* WCFS1 en zijn genoomsequentie beschreven en een kort overzicht van de inhoud van dit proefschrift gepresenteerd, terwijl Hoofdstuk 2 een overzicht gaf van quorum sensing systemen in Gram-positieve bacteriën, die peptiden als signaalmolecuul gebruiken. Het *L. plantarum* genoom codeert voor een groot aantal regulatiegenen (261), waaronder 26 twee-component signaal-sensor en -regulator genen die samen 13 complete TCS koppels vormen. Tevens werden nog een geïsoleerde twee-component signaal-sensor en -regulator op verschillende posities op het genoom aangetroffen. Deze TCS zijn waarschijnlijk betrokken bij de detectie van milieusignalen, maar ook detectie van speciale uitgescheiden quorum sensing signaalmoleculen.

In Hoofdstuk 3 werd de analyse van het hele genoom naar de aanwezigheid van quorum sensing TCS (QS-TCS) in *L. plantarum* beschreven. Vier TCS vertonen sterke gelijkenis met TCS die betrokken zijn bij quorum sensing die peptiden als signaalmolecuul gebruiken, waarvan er drie experimenteel verder werden onderzocht. Deze TCS bleken elk een gen te bevatten voor een potentieel peptide signaalmolecuul. Twee leken voor een eenvoudig peptide zonder modificaties te coderen, waarvan er een identiek was aan een regulatiesysteem dat de productie reguleert van een anti-microbieel peptide in een andere *L. plantarum* stam. Het derde TCS dat werd beschreven in Hoofdstuk 4, vertoont verrassenderwijs gelijkenis met de genetische organisatie van een quorum sensing systeem uit de ziekteverwekkende bacterie *Staphylococcus aureus* (de zogenaamde ziekenhuisbacterie). Moleculair biologische analyse van het *L. plantarum*-systeem gaf aan dat het betrokken is bij aanhechting aan oppervlakken en regulatie van genen coderend voor celoppervlak-eiwitten. Tevens werd een gen gevonden dat codeert voor een klein peptide (slechts 5 aminozuren) met een voor lactobacillen nieuw type chemische modificatie. Het vierde TCS leek erg op dit systeem, maar miste 2 genen. Tenslotte bleek dat de geïsoleerde twee-component sensor en regulator genen beiden ook quorum sensing karakteristieken vertonen. Deze vormen daarom mogelijk een vijfde quorum sensing TCS dat peptiden als signaalmolecuul gebruikt. Naast bovenstaande peptide quorum sensing TCS, gaf genoomanalyse aan dat *L. plantarum* ook een gen bevat dat voor productie van een ander soort signaalmolecuul zorgt (geen peptide), dat in vele verschillende bacteriegroepen wordt aangetroffen. Dit communicatiesysteem zou daarom communicatie met andere bacteriesoorten mogelijk kunnen maken.

In Hoofdstuk 5 tenslotte, werd de experimentele analyse beschreven van een ander type regulator gen dat betrokken bleek te zijn bij regulatie van aanhechting aan oppervlakken en celklontering.

Het wordt verwacht dat met het toenemende aantal bekende genoomsequenties van melkzuurbacteriën en het combineren van klassieke moleculaire biologische methoden en bioinformatica, er ook meer inzicht zal komen in de complexe stofwisselings en regulatienetwerken (waaronder quorum sensing) in melkzuurbacteriën. Daarnaast kunnen we deze nieuwe moleculair biologische methoden nu ook toepassen om de aanwezigheid, activiteit en functies van quorum sensing systemen in melkzuurbacteriën te bestuderen in hun natuurlijke ecosystemen, zoals het maagdarmkanaal en zuivelproducten. Kennis van quorum sensing systemen in humane maagdarmbacteriën (zoals melkzuurbacteriën), die betrokken zijn bij microbe-microbe en gastheer-microbe interacties, kunnen mogelijk ook leiden tot een verbeterd inzicht in het functioneren van het maagdarmkanaal, ziektepreventie, of de ontwikkeling van producten die melkzuurbacteriën bevatten met specifieke gezondheids-bevorderende eigenschappen. Tevens kunnen genetische regulatiesystemen gebaseerd op quorum sensing mechanismen gebruikt worden in industriële productietoepassingen.

Nawoord

Het afronden van een proefschrift is vaak last-minute werk, maar het kiezen van een AIO-plek soms ook. Dit had namelijk een heel ander proefschrift kunnen zijn, als er begin oktober 1999 niet dat laatste telefoontje was geweest om de volgende dag bij Microbiologie langs te komen voor een sollicitatiegesprek. Terwijl ik al moest beslissen voor andere leuke AIO-posities. Cel-cel communicatie bij darmbacteriën klonk echter zo nieuw en uitdagend voor mij, dat de keus toen snel gemaakt was. Het boekje daarover is nu eindelijk af en dat is een hele opluchting voor mij! Dit proefschrift was echter niet tot stand gekomen zonder de hulp van vele collega's, familie en vrienden.

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Curriculum Vitae

Marcus Henricus Johannes (Mark) Sturme werd geboren op 29 januari 1974 te Kessel (voor de limburgers: ut Keverriék). Na het behalen van achtereenvolgens het HAVO en VWO diploma aan het Bouwens van der Boijecollege te Panningen, werd in september 1993 begonnen met de studie Biologie aan de toenmalige Landbouwuniversiteit Wageningen. Moleculair biologische afstudeerprojecten werden verricht aan de dehalogenerende bacterie *Desulfitobacterium dehalogenans* bij het Laboratorium voor Microbiologie en aan RNA-bindende eigenschappen van het Tomato Spotted Wilt Virus kerneiwit bij het Laboratorium voor Virologie, beiden aan de Landbouwuniversiteit Wageningen. Daarna wisten de zwitserse Alpen hem te verleiden om een afstudeerstage te verrichten aan het overheidsinstituut EAWAG te Dübendorf, Zwitserland, waar de regulatie van 2-hydroxybiphenyl-afbraak door *Pseudomonas azelaica* werd bestudeerd. In september 1999 studeerde hij cum laude af als bioloog, waarna hij een maand later als assistent in opleiding begon aan zijn promotieonderzoek bij het Laboratorium voor Microbiologie van Wageningen Universiteit. Dit promotieonderzoek werd nog 8 maanden verlengd via het Wageningen Centre for Food Sciences (WCFS). De resultaten van dit onderzoek staan in dit proefschrift beschreven. In maart 2005 is hij voor een kleine 2 jaar als postdoc begonnen bij het Laboratorium voor Microbiologie aan de karakterisatie van de microbiële gemeenschap en katabole genen betrokken bij afbraak van gechloreerde alifatische koolwaterstoffen in riviersedimenten.

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