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Mini-Review

Making sense of quorum sensing in lactobacilli: a special focus on *Lactobacillus plantarum* WCFS1

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In silico identification criteria were defined to predict if genes encoding histidine protein kinases (HPKs) and response regulators (RRs) could be part of peptide-based quorum sensing (QS) two-component regulatory systems (QS-TCSs) in Firmicutes. These criteria were used to screen HPKs and RRs annotated on the completed genome sequences of *Lactobacillus* species, and several (putative) QS-TCSs were identified in this way. The five peptide-based QS-TCSs that were predicted on the *Lactobacillus plantarum* WCFS1 genome were further analysed to test their (QS) functionality. Four of these systems 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 of *L. plantarum* C11 and was shown to regulate plantaricin production in *L. plantarum* WCFS1. The third TCS was designated *lamBDCA* for Lactobacillus agr-like module, where the *lamD* gene was shown to encode a cyclic thiolactone peptide. The fourth TCS was paralogous to the *lam* system and contained a putative AIP-encoding gene but lacked the *lamB* gene. Finally, a genetically separated orphan HPK and RR that showed clear peptide-based QS characteristics could form a fifth peptide-based QS-TCS. The predicted presence of multiple (peptide-based) QS-TCSs in some lactobacilli and in particular in *L. plantarum* might be a reflection of the ability of these species to persist in a diverse range of ecological niches.

Two-component regulatory systems in lactic acid bacteria

The lactic acid bacteria (LAB) comprise a diverse group of Gram-positive bacteria that are applied in the production of fermented food products such as dairy, meat and vegetable products (Caplice & Fitzgerald, 1999). Several LAB are also found in the gastrointestinal tracts of humans and other animals (Vaughan *et al.*, 2002). Among the LAB the genus *Lactobacillus* forms a large and diverse group, and several *Lactobacillus* strains are considered to exert health-promoting effects in man and animals (Ouwehand *et al.*, 2002). The genome sequences of a number of *Lactobacillus* species from different ecological niches are currently available (Makarova *et al.*, 2006; Makarova & Koonin, 2007; Siezen *et al.*, 2004). This enables a comparative genomics analysis of lactobacilli that are restricted to a specific niche and have limited physiological abilities, such as *Lactobacillus johnsonii* in the human gastrointestinal tract (Pridmore *et al.*, 2004) and *Lactobacillus delbrueckii* subsp. *bulgaricus* in dairy products (Van de Guchte *et al.*, 2006), with more adaptable species such as *Lactobacillus plantarum* (Kleerebezem *et al.*, 2003), which is found in fermented food products, on plant material (Caplice & Fitzgerald, 1999) and as a natural inhabitant of the human gastrointestinal tract (Ahrne *et al.*, 1998). To allow for efficient colonization and persistence or effective adaptation to changing environmental conditions, lactobacilli require sensory systems to detect (specific) environmental signals. In bacteria, this function is commonly mediated by two-component regulatory systems (TCSs), which consist of a membrane-located histidine protein kinase (HPK) that monitors one or more environmental factors, and a cytoplasmic response regulator (RR), which modulates expression of specific genes. The HPK and RR

Abbreviations: AIP, autoinducing peptide; HPK, histidine protein kinase; QS, quorum sensing; RR, response regulator; TCS, two-component system; TMS, transmembrane segment.

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function as a phospho-relay signal-transduction system (Fig. 1), and the genes encoding the cognate HPK and RR are generally organized in an operon structure (Hoch & Silhavy, 1995). TCSs monitor and respond to changes in environmental conditions such as osmolarity, nutrient availability (C, N, P) or temperature (Hoch & Silhavy, 1995). However, in Gram-positive species, TCSs are also known to respond to specific secreted signalling molecules involved in quorum sensing (QS) (Sturme *et al.*, 2002).

QS-TCSs in Gram-positive bacteria regulate the expression of genes involved in diverse functions such as virulence, competence or bacteriocin production (Kleerebezem *et al.*, 1997). This modulation is done in a coordinated and cell-density-dependent manner, using specific autoinducing signalling peptides (AIPs) that are often post-translationally modified and exported by dedicated transport systems (Ansaldi & Dubnau, 2004; Håvarstein *et al.*, 1995; Zhang & Ji, 2004), and sensed by responsive cells via dedicated HPKs. Bacteria may contain multiple QS-TCSs, underlining the importance of intercellular communication.

This mini-review describes *in silico* criteria for the identification of (putative) peptide-based QS-TCSs in lactobacilli and other Firmicutes. In addition, it describes the application of those criteria in the identification of QS-TCSs in the model organism *L. plantarum* WCFS1 (Kleerebezem *et al.*, 2003). Detailed analysis and experimental confirmation of the functionality of predicted peptide-based QS-TCSs in *L. plantarum* WCFS1 is also discussed.

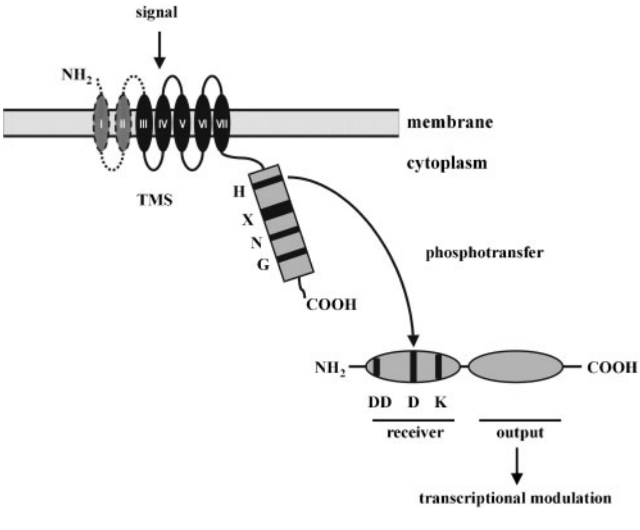

Fig. 1. General signal transduction mechanism of two-component regulatory systems. The cellular localization and sequence motifs characteristic of two-component histidine protein kinases of the HPK_{10} subfamily (membrane-bound) and response regulators (cytoplasmic) are depicted (see also Table 1). TMS, transmembrane 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.

Protein architecture of peptide-based QS-TCSs

A distinctive functional feature of TCSs is the ability to transfer a phosphoryl group from ATP to a receptor protein. As a result, the two 'components' of the system contain characteristic functional domains. The HPKs have a conserved C-terminal ATP-binding domain in which the phosphoryl-accepting histidine residue is located, and related to that they also have highly conserved clusters of residues called homology boxes (Grebe & Stock, 1999; Parkinson & Kofoid, 1992). Based on the presence and structure of the various homology boxes, a comprehensive classification of HPKs was made by Grebe & Stock (1999). In this classification the vast majority of peptide-based QS-TCSs comprise specific HPKs belonging to the subfamily called HPK_{10}. The only exception known to date is ComP of *Bacillus subtilis*, which is a QS-HPK that belongs to a different HPK subfamily (HPK_7). The HPK_{10} homology boxes have the following characteristics: the H-box (histidine phosphorylation site) contains a tyrosine two residues downstream from the conserved histidine (characteristic motif F+HDYxN) and lacks an otherwise conserved proline residue at position 5 downstream; the X-box (a hydrophobicity pattern conserved in several subfamilies) is present; the N-box has only one conserved asparagine residue (characteristic motif DNAIE); and the G-box, which plays a critical role in phosphoryl transfer, has a characteristic FSTKGxGxGLGL motif (Fig. 1). The D-box that in most subfamilies is part of the nucleotide-binding domain is absent in this subfamily. Furthermore, HPK_{10} subfamily members commonly possess five to seven N-terminal transmembrane segments (TMSs) (Fig. 1).

In general, RRs contain a C-terminal DNA-binding domain and a N-terminal CheY-like receiver domain (REC: SM00448) that includes a conserved phosphoryl-accepting aspartate residue (Volz, 1993) (Fig. 1). Analogous to the HPKs, the RRs have been classified based on the receiver and the DNA-binding domains (Grebe & Stock, 1999). In both classifications the RRs related to HPKs of the HPK_{10} subfamily comprise a separate subfamily: the RD and ComE subfamily, respectively. Most established QS-RRs are encompassed within this RD/ComE subfamily of RRs. However, in accordance with the unusual sequence of its cognate HPK, the competence-regulating RR (ComA) of *B. subtilis* belongs to the RE rather than the RD subfamily of RRs and contains a HTH-LuxR DNA-binding domain (SM00421) (Fuqua *et al.*, 1994). In a more recent analysis most RRs of the RD/ComE subfamily were classified in the LytTR family of response regulators (PF04397), based on a conserved motif in the C-terminal helix–turn–helix (HTH) DNA-binding domain (Nikolskaya & Galperin, 2002). For RRs the presence of a HTH-LytTR DNA-binding domain does not in all cases classify a RR as a peptide-based QS-RR (Nikolskaya & Galperin, 2002). However, the presence of an adjacent HPK with HPK_{10}-subfamily characteristics can be used to classify the RR as such.

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Genomic context: linkage of peptide-based QS-TCSs with AIPs and other QS-related functionalities

In Gram-positive bacteria, genes encoding peptide-based QS-TCSs are in general preceded by genes encoding the cognate autoinducing peptide (AIP). Many AIPs have a structure similar to class I bacteriocins (lantibiotics) (McAuliffe *et al.*, 2001) or to class II bacteriocins (non-lantibiotics) (Ennahar *et al.*, 2000). These AIPs contain recognizable leader peptides with conserved residues, such as the double-glycine leader peptides of most class II and some class I bacteriocins (the consensus for residues -12 to -1 of GG-leader is LSxxELxxIxGG) (Nes & Eijsink, 1999). The web-based bacteriocin genome-mining tool BAGEL (http://bioinformatics.biol.rug.nl/websoftware/bagel/bagel_start.php) might be used to identify such bacteriocin-like AIPs that contain leader peptides (de Jong *et al.*, 2006). In addition, some QS-TCS genes are genetically linked to genes encoding AIP transport and/or modification proteins, bacteriocins and bacteriocin-immunity proteins (Kleerebezem *et al.*, 1997). For double-glycine-type AIPs the cognate transporters are ABC transporters that contain a characteristic N-terminal peptidase C39 domain (COG2274) (Håvarstein *et al.*, 1995), while in the case of *agr*-like systems AgrB-type cysteine proteases are involved in transport and modification of AIPs (Nakayama *et al.*, 2006; Qiu *et al.*, 2005; Zhang & Ji, 2004).

In silico identification of candidate QS-TCSs in lactobacilli

To identify new putative QS-TCSs, protein sequences of experimentally verified QS-TCSs in *Staphylococcus aureus* (AgrCA) (Ji *et al.*, 1997) and *L. plantarum* C11 (PlnBCD) (Diep *et al.*, 1996) were collected from public databases (http://www.ncbi.nlm.nih.gov/). Potential system homologues were collected from the genomes of lactobacilli and other Firmicutes via iterative BLASTP searches for HPKs or RRs, using default settings (PSI-BLAST, *E*-value threshold 1×10^{-5}) (Altschul *et al.*, 1990).

Then, various peptide-based QS-specific protein characteristics were used to reduce the list of homologues. These characteristics included: (i) for HPKs the presence of HPK_{10}-subfamily domains (Grebe & Stock, 1999) and five to seven N-terminal transmembrane segments (TMSs), and (ii) for RRs the presence of RD/ComE subfamily domains (Grebe & Stock, 1999) or HTH_LytTR DNA-binding domains (Nikolskaya & Galperin, 2002). In addition, (iii) the presence of adjacent AIP-like genes and additional peptide-based QS-related genes was investigated for these putative QS-TCSs. Protein domains were predicted using the HMMs of SMART, including outlier homologues and PFAM domains (Schultz *et al.*, 1998), and membrane topology was predicted using TMHMM 2.0 (Krogh *et al.*, 2001). The remaining HPK and RR sequences were aligned, and bootstrapped neighbour-joining trees were constructed with CLUSTAL_X (Thompson *et al.*, 1997). The trees were analysed and phylogenetic relationships between sequences established (Van der Heijden *et al.*, 2007). The results for lactobacilli are summarized in Table 1 and in Supplementary Figs S1 and S2 (available with the online version of this paper).

The *in silico* analysis confirmed previously described QS-TCSs and identified novel putative QS-TCSs and AIPs in lactobacilli. The analysis predicted the presence of five QS-TCSs in *L. plantarum* WCFS1, two QS-TCSs in both the intestinal species *Lactobacillus acidophilus* NCFM and *L. johnsonii* NCC533, one QS-TCS in the intestinal species *L. salivarius* subsp. *salivarius* UCC118 and the food species *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365, and no QS-TCS in the intestinal species *L. gasseri* ATCC 33323. In most cases these putative QS-TCSs had adjacent genes encoding class II bacteriocin-like or lantibiotic-like peptides, which might serve as AIPs, as well as ABC transporters with a peptidase C39 domain, which supports a role in QS-regulated bacteriocin production (see Fig. S2). The QS-functionality of *abpIPKR* in *L. salivarius* and LBA1798-LBA1800 in *L. acidophilus* was previously shown (Flynn *et al.*, 2002; Dobson *et al.*, 2007), and the functional analysis of the putative QS-TCSs in *L. plantarum* is discussed in the next section.

In addition, in each of the food species *Lactobacillus brevis* ATCC 367, *L. casei* ATCC 334 and *L. sakei* subsp. *sakei* 23K one putative QS-TCS was identified. However, for those systems the functionality of the predicted QS-TCS is doubtful, since their HPK genes seemed to be (i) interrupted (internal deletions) or (ii) incomplete (N-terminus absent), or (iii) the cognate HPK was apparently absent (i.e. not adjacent to the RR). A good example of this is the *sppIPKR* QS system, which was detected and functional in several *L. sakei* strains, but not functional in *L. sakei* subsp. *sakei* 23K (Møretrø *et al.*, 2005). This is caused by a 4 bp internal deletion in the HPK gene (*sppK*) of this strain, resulting in two truncated HPK fragments (*sppkN* and *sppKC*) and thereby a non-functional QS-TCS. In *L. casei* ATCC 334 there are two genes present encoding RRs with RD and LytTR domains. One appears to be an orphan gene (LSEI_2389) with a putative AIP downstream (LSEI_2390). The other RR (LSEI_2599) is part of a putative QS-TCS, where the HPK encoded by LSEI_2600 contains the HPK_{10}-subfamily motifs but lacks most of the N-terminus with TMS. Finally, for *L. brevis* ATCC 367 there are two HPKs present (typical is only one HPK) downstream of a RR with RD and LytTR domains (LVIS_0163), but they lack either a clear H-box (LVIS_0164) or N-box (LVIS_0165), which are typical of the HPK_{10} subfamily.

Functional analysis of candidate QS-TCSs in *L. plantarum* WCFS1

Previous annotation of the 3.3 Mb genome sequence of *L. plantarum* WCFS1 revealed the presence of 13 genetically linked TCSs, and one orphan HPK and RR (Kleerebezem

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Table 1. General features of HPKs and RRs of candidate QS-TCSs in *L. plantarum* WCFS1 and related lactobacilli (see also Figs S2 and S3)

Accession numbers for *Lactobacillus* genomes: *L. plantarum* WCFS1 (AL935263); *L. acidophilus* NCFM (CP000033); *L. johnsonii* NCC533 (AE017198); *L. salivarius* subsp. *salivarius* UCC118 (CP000233); *L. sakei* subsp. *sakei* 23K (CR936503); *L. brevis* ATCC 367 (CP000416); *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (CP000412); *L. casei* ATCC 334 (CP000423).

Gene*/ accession no.	Locus†	Size (aa)	TMS‡	HPK$_{10}$ subfamily§	Gene*/ accession no.	Locus†	Size (aa)	LytTR domains	Gene*/ accession no.	Locus†
HPK					**RR**				**AIP**	
***L. plantarum* WCFS1 (3.34 Mb)**										
plnB	lp_0416	442	7‖	+	*plnC*/*plnD*	lp_0417/ lp_0418	247	+	*plnA*	lp_0415
pltK	lp_1355	420	6	+	*pltR*	lp_1356	255	+	*pltA*	lp_1354a
hpk4	lp_1488	343	−	− (HPK$_7$)	*rrp4*	lp_1487	217	− (LuxR)		
hpk6	lp_1943	367	5	− (HPK$_7$)	*rrp6*	lp_1942	201	− (LuxR)		
hpk9	lp_3063	422	6	+	*rrp8*	lp_2665	249	+		
hpk10	lp_3088	416	6	+	*rrp10*	lp_3087	248	+	−	lp_3089
lamC	lp_3581	419	5	+	*lamA*	lp_3580	247	+	*lamD*	lp_3581a
***L. acidophilus* NCFM (2.0 Mb)**										
YP_193512	LBA0602	426	6	+	YP_193513	LBA0603	265	+		
YP_194634	LBA1799	440	7	+	YP_194633	LBA1798	274	+	YP_194635	LBA1800
***L. johnsonii* NCC533 (2.0 Mb)**										
NP_964473	LJ0448	419	6	+	NP_964474	LJ0449	255	+		
NP_964617	LJ0764	435	6	+	NP_964619	LJ0766	265	+	NP_964616	LJ0763b
***L. salivarius* subsp. *salivarius* UCC118 (2.13 Mb)**										
abpK	LSL_1913	429	7	+	*abpR*	LSL_1912	264	+	*abpIP*	LSL_1914
***Lactobacillus sakei* subsp. *sakei* 23K (1.9 Mb)**										
sppKN+*sppKC*	LSA0561 +LSA0562	$69^{N,\Delta}$ $+183^{N,\Delta}$	0	+	*sppR*	LSA0563	248	+	*sppIP*	LSA0560_b
***L. brevis* ATCC 367 (2.35 Mb)**										
YP_794364	LVIS_0164	444	6	± (no H-box)	YP_794363	LVIS_0163	251	+		
YP_794365	LVIS_0165	443	7	± (no N-box)						
***L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (1.9 Mb)**										
YP_812154	LBUL_0022	434	6	+	YP_812153	LBUL_0021	260	+		
***L. casei* ATCC 334 (2.93 Mb)**										
					YP_807570	LSEI_2389	268	+	YP_807571	LSEI_2390
YP_807763	LSEI_2600	303^{N}	2	+	YP_807762	LSEI_2599	234	+		

*, †Gene name and locus number from http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi.

‡TMS, transmembrane segments as predicted from TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

§HPK classification after Grebe & Stock (1999).

‖Experimentally confirmed data for PlnB in *L. plantarum* C11 (Johnsborg *et al.*, 2003).

^{N}, Incomplete gene: N-terminal domain (partially) missing.

$^{\Delta}$, 4 bp deletion in *sppK* gene (Møretrø *et al.*, 2005).

et al., 2003). Out of these, five TCSs were identified as candidate QS-TCSs based on the *in silico* approach described above. They were predicted to comprise five HPKs that showed characteristics of the HPK$_{10}$ subfamily and six RRs that contained a HTH-DNA-binding domain of the LytTR family. Of the remaining TCSs, which did not fit the general *in silico* criteria for peptide-based QS-TCSs, we found on closer examination that there were two TCSs where the HPKs and RRs showed similarity to another type of QS-TCS, as found for the ComPA system of *B. subtilis.* These HPKs showed HPK$_7$-subfamily characteristics (Grebe & Stock, 1999) and the RRs contained a HTH-DNA-binding domain of the LuxR family (Fuqua *et al.*, 1994) (Table 1).

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Four adjacent HPK- and RR-encoding genes constituted complete TCSs that were classified as candidate peptide-based QS-TCSs (*pln*, *plt*, TCS10 and *lam*). In addition, the orphan HPK and RR could constitute a fifth peptide-based QS-TCS. The relevant features of these HPKs and RRs are summarized in Table 1 and their genetic organization is shown in Supplementary Figs S2 and S3. For all of the complete QS-TCSs a putative AIP was encoded upstream (Fig. 2 and Fig. S3), two of which (*pln* and *plt*) contained a putative double-glycine-type leader peptide, while the third and fourth were of a different type. The gene connected to the *lam* QS-TCS (*lamD*) encodes a cyclic thiolactone AIP (Sturme *et al.*, 2005), whereas the one connected to TCS10 encodes a putative AIP that shows little similarity to known AIPs. Details of the characterization and functionality of these putative QS-TCSs are discussed below.

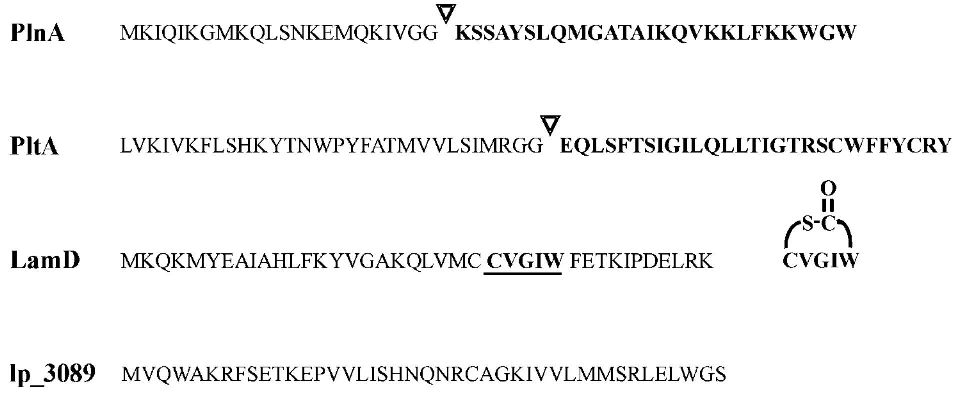
Plantaricin TCS *pln* (lp_0415 to lp_0418)

The plantaricin TCS *pln* (lp_0415 to lp_0418) was identical to the previously described *plnABCD* system of *L. plantarum* C11, which regulates the production of class II antimicrobial peptides (AMPs) (Diep *et al.*, 1996). This system contains a gene (*plnB*: lp_0416) encoding a typical HPK of subfamily HPK_{10} that was shown to have seven TMSs (confirmed in strain C11 by Johnsborg *et al.*, 2003). Downstream of *plnB*, two RRs are encoded (*plnC* and *plnD*) that both contain the RD receiver domains and LytTR HTH-DNA-binding domains. Upstream of *plnB*, *plnA* encodes a 48 amino acid double-glycine-type AIP precursor (Nes & Eijsink, 1999). Genes encoding class II bacteriocins (PlnE-PlnF, PlnJ-PlnK and PlnN) were localized near the *pln* regulatory module (Supplementary Fig. S3). Cleavage of the double-glycine-type leader peptide from the PlnA precursor peptide results in a linear AIP of 26 amino acids without modifications and an amphipathic character (Fig. 2). The *pln* system was found to be functional in *L. plantarum* WCFS1, as was shown by a bacteriocin agar-well diffusion assay with *L. plantarum* 965 as an indicator strain (Fig. 3). The *L. plantarum* WCFS1 native state was bacteriocin-negative (Bac^{-}), but bacteriocin production could be induced with either bacteriocin-positive supernatant of strain C11 (Bac^{+}) or purified PlnA peptide.

The *pln* system of WCFS1 is expected to play a role in competition with other bacteria, as in strain C11 it regulates the production of the bacteriocins PlnE-PlnF, PlnJ-PlnK and PlnN. These C11 plantaricins showed activity against closely related species (Anderssen *et al.*, 1998), which can be found in the same ecological niches as *L. plantarum* strains WCFS1 and C11 (Vaughan *et al.*, 2002). However, some strain-specific differences in plantaricin activity and target specificity might be expected for various *L. plantarum* strains, considering the differences in *pln* gene composition and sequence variations identified in the *pln* operons of strains WCFS1, C11 and NC8 (Maldonado *et al.*, 2004; Molenaar *et al.*, 2005).

TCS *plt* (lp_1354a, lp_1355 and lp_1356)

The *plt* locus (lp_1354a, lp_1355 and lp_1356) encodes a typical HPK (*pltK*: lp_1355) of the HPK_{10} subfamily with five or six predicted TMSs, and a RR (*pltR*: lp_1356) that contains a RD-type receiver domain and a LytTR HTH-DNA-binding domain. Upstream of *pltK*, a 58 amino acid double-glycine-type AIP precursor appears to be encoded (*pltA*: lp_1354a). The predicted mature PltA peptide is a 28 amino acid candidate AIP that is expected to be unmodified (Fig. 2). Northern blot analysis showed that the *pltAKR* operon is transcribed as a single polycistronic, 2.4 kb transcript in a cell-density-dependent manner (Fig. 4). The predicted mature PltA peptide was chemically



PlnA MKIQIKGMKQLSNKEMQKIVGG ▽ **KSSAYSLQMGATAIKQVKKLFKKWGW**

PltA LVKIVKFLSHKYTNWPYFATMVVLSIMRGG ▽ **EQLSFTSIGILQLLTIGTRSCWFFYCRY**

LamD MKQKMYEAIAHLFKYVGAKQLVMC **<u>CVGIW</u>** FETKIPDELRK

lp_3089 MVQWAKRFSETKEPVVLISHNQNRCAGKIVVLMMSRLELWGS

Fig. 2. AIPs encoded on the *L. plantarum* WCFS1 genome. Triangles indicate the cleavage site between the double-glycine leader peptide and the (predicted) mature peptide in PlnA and PltA (shown in bold). The underlined bold residues in the LamD precursor peptide are processed to the mature thiolactone peptide shown on the right. For the putative AIP encoded by lp_3089 the mature peptide sequence and structure are unknown.

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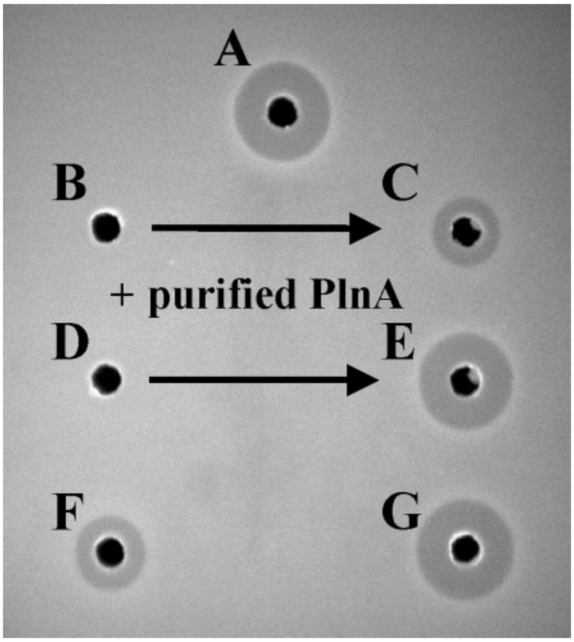

Fig. 3. Agar well diffusion assay for plantaricin production in *L. plantarum* WCFS1 and the related strain C11, using indicator strain *L. plantarum* 965. To induce plantaricin production in *L. plantarum* WCFS1, this strain was grown in MRS medium containing 1 μg ml^{-1} of purified PlnA peptide or in MRS medium containing 2 % of spent culture supernatant of bacteriocin-producing *L. plantarum* C11. To obtain a bacteriocin-negative derivative of *L. plantarum* C11, bacteriocin-producing cells of this strain were inoculated in MRS at a density below 10^4 c.f.u. ml^{-1} (previously reported to result in loss of plantaricin production). 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 $<10^4$ c.f.u. ml^{-1} dilution; E, Pln production in C11 ($<10^4$ c.f.u. ml^{-1} 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, indicator strain *L. plantarum* 965 and purified PlnA were generous gifts from Dzung Diep and Ingolf Nes, Norwegian University of Life Sciences, Ås, Norway.

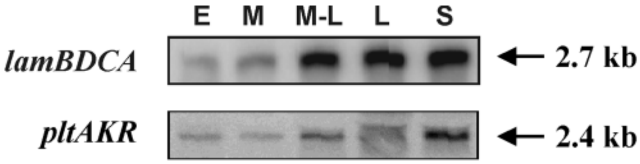

Fig. 4. Northern blot analysis of temporal gene expression of the *lamBDCA* and *pltAKR* operons of *L. plantarum* WCFS1, when grown in MRS at 30 °C, without agitation. Growth phases are indicated above the samples: E, M and L indicate early-, mid- and late-exponential growth phases and S the stationary growth phase. Equal amounts of RNA (10 μg) were loaded. Expression of *lamBDCA* was monitored using a *lamC* internal probe, and expression of *pltAKR* using a *pltK* internal probe. Transcript sizes are shown on the right.

synthesized, but was water-insoluble and acted as a gel above 35–40 % purity. Therefore it could not be used in induction experiments. The water insolubility could result from the high hydrophobicity and lack of α-helical amphipathic characteristics of this peptide, which is typical in class II bacteriocin-like AIPs (Anderssen *et al.*, 1998). Alternatively, the *pltA*-encoded peptide could be subject to unpredicted post-translational modifications that affect its solubility and render a functional secreted AIP. In conclusion, the role of the proposed (modified) mature PltA peptide in the cell-density-dependent expression of the *pltAKR* locus and possible secondary target loci remain to be established. Nevertheless, its canonical genetic organization suggests a role of the *pltAKR* operon in QS, which is supported by its observed cell-density-dependent expression pattern. The *plt* locus does not appear to be genomically directly linked to any ABC transporter or bacteriocin-encoding gene (Supplementary Figs S2 and S3). However, the PltA pre-peptide could be transported by another ABC transporter with a peptidase C39 protease-family domain (Håvarstein *et al.*, 1995), such as the ABC transporter encoded within the *pln* locus (encoded by *plnG* and *plnH*: Fig. S3).

TCS *lam* (lp_3580 to lp_3582)

The TCS encoded by lp_3580 to lp_3582 was designated *lam* for *Lactobacillus agr*-like module, as it showed a similar gene organization to the *agrBDCA* QS system of *S. aureus* (Ji *et al.*, 1995). The *L. plantarum lamBDCA* locus encodes a HPK (*lamC*: lp_3581) of the HPK_{10} subfamily with five predicted TMSs, and a RR (*lamA*: lp_3580) that contains a RD-type receiver domain and a LytTR HTH-DNA-binding domain. The locus is organized as an operon and is transcribed as a single transcript in a cell-density-dependent manner (see Fig. 4 and Sturme *et al.*, 2005). Analogous with the staphylococcal *agr* system (Ji *et al.*, 1995), the *L. plantarum lamBDCA* locus is involved in production of a cyclic thiolactone peptide (CVGIW) which is predicted to derive from the LamD precursor (lp_3581a) that is processed by LamB (lp_3582) (Fig. 2). Analysis of a *lamA* mutant revealed a role for the *lam* operon in *L. plantarum* biofilm-forming capacity. Transcriptome profiling of wild-type and *lamA* mutant strains in early-, mid- and late-exponential phase uncovered only a small set of clustered genes (2 % of all genes) that were significantly modulated by the *lamA* mutation. These data confirmed the autoregulation of *lamBDCA*, and showed the regulation of a surface polysaccharide biosynthesis gene-cluster, and several cell envelope and sugar utilization functions (Sturme *et al.*, 2005). The same study suggested that a direct repeat sequence within the *lamB* promoter region (5′-TCTTTAAAT – 12 bp – TCTTAAAA-3′) that displays similarity with previously established cognate *cis* elements of LamA homologues (Morfeldt *et al.*, 1996; Qin *et al.*,

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2001; Quadri *et al.*, 1997; Risøen *et al.*, 2000) acts as the LamA DNA-binding site.

TCS10 (lp_3087 and lp_3088)

The bioinformatic analysis suggests that the HPK and RR of TCS10 (lp_3087 and lp_3088) are inparalogues of the *lamCA*-encoded proteins, with the cognate HPKs and RRs showing 55 % and 70 % identity, respectively, at the amino acid level. This module lacks a *lamB* homologue and originally seemed to lack a *lamD* homologue. However, a region somewhat upstream of the *hpk10* gene is remarkably similar to that of the region upstream of the *lam* system. This (promoter) region contains a direct repeat (5′-TCTTGAAAT – 12 bp – TCTTAAAG-3′), displaying very high similarity with the proposed regulatory element of the *lamB* promoter (see above). Interspersed between this regulatory region of TCS10 and the *hpk10* gene is a region of about 130 nucleotides that on closer inspection was shown to encode a small peptide (lp_3089) that initially was not annotated (Fig. 2 and Supplementary Fig. S3). Although the peptide shows some conservation with respect to the *lamD* gene product, it is not clear whether it is a genuine AIP. Taken together these findings support an inparalogous relationship between the RRs and HPKs of the TCS10 and *lam* systems, and suggests that there could be cross-talk between these two regulatory systems.

Orphan genes *rrp8* (lp_2665) and *hpk9* (lp_3063)

The two orphan *hpk* and *rrp* genes show typical characteristics of peptide-based QS systems. The orphan gene *hpk9* (lp_3063) was predicted to encode a HPK_{10}-subfamily protein containing six TMSs. Interestingly, immediately downstream (7 bp) of *hpk9* a small ORF is located (lp_3062) that shows 46 % homology to the C-terminal domain of the TfoX protein of *Haemophilus influenzae* (PF04994). The TfoX protein is proposed 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 (Zulty & Barcak, 1995). 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 fulfil this function, as it was not transcriptionally linked and lacks a RD-type receiver domain and contains a typical HTH-AraC DNA-binding domain (SM00342) rather than the canonical LytTR or HTH-LuxR DNA-binding domains (see Supplementary Fig. S3). It is possible that the *hpk9*-encoded HPK in combination with the *rrp8*-encoded orphan RR forms a functional TCS, which could be involved in peptide-based QS. This possibility is supported by the finding that *rrp8* (lp_2665) encodes a RR with a typical LytTR HTH-DNA-binding domain. Moreover, the neighbour-joining trees based on protein sequence alignments of HPKs and RRs belonging to QS-TCS showed that the *hpk9*- and *rrp8*-encoded proteins (LPL03063 and LPL02665 in Supplementary Fig. S1) cluster with several HPKs and RRs of other Firmicutes that are adjacent on their respective genomes, such as LEUM_0009/LEUM_0008 from *Leuconostoc mesenteroides* or EF1820/EF1822 from *Enterococcus faecalis*. Interestingly, the gene downstream of *rrp8* (lp_2664) contains a HDc-superfamily-type phosphohydrolase-domain (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.

Additional candidate QS-TCSs and genes involved in non-peptide-based QS

Besides the typical peptide-based QS-TCSs, TCS4 (lp_1487 and lp_1488) and TCS6 (lp_1942 and lp_1943) both encode HPK_7-subfamily-type HPKs (Grebe & Stock, 1999) adjacent to a RR containing a HTH-LuxR DNA-binding domain (see Table 1 and Supplementary Fig. S3). This resembles the protein architecture of the ComPA QS-TCS of *B. subtilis* that is involved in QS regulation of competence (Weinrauch *et al.*, 1989, 1990). However, both the HPK_7 subfamily and the HTH-LuxR DNA-binding domain are not exclusively associated with QS-TCSs (Gray & Garey, 2001; Grebe & Stock, 1999), indicating that on the basis of *in silico* analysis the involvement of TCS4 and TCS6 in QS can only be tentatively suggested. Interestingly, next to the putative QS-TCS an isolated homologue of the *luxS* gene was identified (lp_0774), encoding the autoinducer-2 (AI-2) synthase (Schauder *et al.*, 2001). AI-2 is thought to play a role in QS, although there is still some debate on its actual physiological role (Sun *et al.*, 2004). Associated functions like *lsr*, which is involved in AI-2 uptake in *Salmonella typhimurium*, or *luxPQ* involved in AI-2 sensing and signal transduction in *Vibrio harveyi*, were not identified in the *L. plantarum* genome (Sun *et al.*, 2004). AI-2 could be involved in interspecies communication of *L. plantarum* with other bacteria within the same niche, as the *luxS* gene was detected in both Gram-negative and Gram-positive bacteria (Xavier & Bassler, 2003). Interestingly, AI-2 activity was detected in rumen samples (Mitsumori *et al.*, 2003), suggesting a natural role of the AI-2 QS system in gastrointestinal ecosystems.

Concluding remarks

L. plantarum WCFS1 contains a relatively high number of (putative) peptide-based QS-TCSs (five, of which at least four can be directly coupled to a putative AIP), as well as other putative QS genes. This could reflect the ecological flexibility of this species, which can be found in plants, fermented foods and the gastrointestinal tract. In comparison, the genomes of related lactobacilli that are more restricted to specific environments seem to encode significantly fewer peptide-based QS-TCSs, despite the differences in genome size (see Table 1). Depending on

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niche-specific conditions, different signalling systems might be triggered, resulting in a gene-regulatory network that controls a variety of phenotypic traits in response to environmental conditions in combination with cell density. The potential for cross-talk between the *lamBDCA* and TCS10 systems might be exemplary for the complexity of a regulatory network that controls surface adherence of *L. plantarum* under specific conditions (Sturme *et al.*, 2005). Moreover, the presence of competing micro-organisms could activate specific QS-TCSs involved in competition, as has been shown for the plantaricin system in *L. plantarum* NC8 (Maldonado *et al.*, 2004). Interestingly, the involvement of *agr*-like TCSs in host–microbe interactions of commensal bacteria has recently been suggested for *Roseburia inulinivorans* (Scott *et al.*, 2006).

Further experimental studies on the regulatory mechanisms of the different QS systems of *L. plantarum* and other *Lactobacillus* species and their effects on (global) gene regulation will be necessary, to provide more insight into the role of these systems in the survival of these organisms in their natural environments.

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