Six putative two-component regulatory systems isolated from Lactococcus lactis subsp. cremoris MG1363

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INTRODUCTION

Bacteria are able to sense and respond to a variety of environmental stimuli. Reception and transmission of these environmental signals to effect the appropriate physiological adaptation often involves so-called two-component systems (2CSs), which usually consist of a sensor or histidine protein kinase (HPK) and an effector or response regulator (RR) (for reviews, see Stock et al., 1989, 1990; Parkinson & Kofoid, 1992; Parkinson, 1993; Mizuno, 1998; Fabret et al., 1999). Typically, the HPK, which is often anchored to the cytoplasmic membrane, monitors an environmental parameter(s) whereas the corresponding cytoplasmic RR mediates an adaptive response to this environmental signal, in many cases a change in gene expression. Communication between a HPK and its cognate RR occurs through phosphorylation and dephosphorylation reactions. The underlying molecular mechanism of 2CS signal transduction therefore appears to be simple. However, much more complicated devices have been described which integrate multiple environmental signals and also ensure that output response is smoothly graded with stimulus intensity (for a review, see Egger et al., 1997).

The HPK and RR proteins are composed of distinct domains or modules which can be arranged in different configurations to build signalling circuits (for reviews, see Stock et al., 1989; Parkinson & Kofoid, 1992; Parkinson, 1993; Alex & Simon, 1994; Egger et al., 1997). In the simplest circuit, the HPK is composed of a C-terminal transmitter module attached to an N-terminal signal-input domain, whilst the RR consists of an N-terminal receiver module connected to a C-terminal signal-output domain. Another phospho-transfer signalling device, referred to as a histidine phosphotransfer protein (HPT) (Ishige et al., 1994) consists of approximately 120 amino acids and contains...
a short consensus motif of approximately 25 amino acids including an invariant histidine residue, which can acquire a phosphoryl group from a cognate HPK and/or RR and transmits this phosphoryl group to the receiver domain of the appropriate RR. Hybrid or unorthodox HPK proteins have also been described (Parkinson & Kofoid, 1992), some of which harbour the signal-input and output domains, as well as the transmitter and receiver modules within one protein.

To date, members of the HPK and RR families have been described for over fifty different bacterial species and for several eukaryotic organisms, including Neurospora crassa, Saccharomyces cerevisiae, Arabidopsis thaliana and Dictyostelium discoideum (Alex & Simon 1994; Chang & Meyerowitz, 1994; Appleby et al., 1996; Pao & Saier, 1997; Loomis et al., 1997) and it can be inferred that such systems may be ubiquitous in many living organisms. An apparent exception to this rule is the methanogenic archaeon, Methanococcus jannaschii (Bult et al., 1996).

In Gram-positive bacteria, several processes are regulated by 2CSs. Analysis of the genome sequence of Bacillus subtilis identified the presence of 36 HPKs and 34 RRs. Several 2CSs in B. subtilis have been extensively studied, these include those involved in chemotaxis (CheA-CheY), phosphate metabolism (PhOR-PhOP), anaerobic growth (ResE-ResD), competence development (ComP-ComA) and degradative enzyme production (DegS-DegU) (Fabret et al., 1999). Quorum sensing mechanisms in Gram-positive bacteria are also regulated by 2CSs but in a cell-density-dependent manner. These include the development of genetic competence in B. subtilis (Dubnau, 1991) and Streptococcus pneumoniae (Pestova et al., 1996), virulence responses in Staphylococcus aureus (Morfeldt et al., 1996) and the production of antimicrobial peptides by lactic acid bacteria (Diep et al., 1994, 1996; Kuipers et al., 1993, 1995; Quadri et al., 1997; Van der Meer et al., 1993; O’Keefe et al., 1999). In each case, a secreted peptide functions as the input signal for a specific sensor of a 2CS. The genes which encode the precursor of the peptide and genes encoding the proteins involved in the 2CS and/or those involved in the secretion of the peptide pheromone are transcriptionally linked and the synthesis of the peptide pheromones appears to be an autoregulatory process. Another common feature of these systems is the involvement of an ATP-binding cassette (ABC) exporter in the secretion of the peptide pheromone. A plasmidborne 2CS which regulates copper resistance in Lactococcus lactis has also been described (Khunjakr et al., 1999).

Previously, we reported the identification of five HPKs from L. lactis MG1363 by complementation of HPK-deficient Escherichia coli mutants (O’Connell-Motherway et al., 1997). This study reports on the identification and sequence of the five cognate RR genes and identification of a novel 2CS from L. lactis by a PCR approach, as well as preliminary characterization of the six putative 2CSs from L. lactis MG1363.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** Strains and plasmids used in this study are listed in Table 1. L. lactis was cultivated at 30 °C (unless otherwise indicated) in M17 broth (Terzaghi & Sandine, 1973) containing 0.5% (w/v) glucose, TYG broth (O’Sullivan, 1996) or minimal medium (Molenaar et al., 1993). E. coli strains were grown in Luria–Bertani (LB) broth at 37 °C with agitation (Sambrook et al., 1989).

When required, growth media contained ampicillin (100 µg ml⁻¹), chloramphenicol (20 µg ml⁻¹), erythromycin (50 µg ml⁻¹), kanamycin (45 µg ml⁻¹), tetracycline (12.5 µg ml⁻¹) or vancomycin (50 µg ml⁻¹). X-Gal and 5-bromo-4-chloro-3-indolyl phosphate (X-P) were used at final concentrations of 40 µg ml⁻¹.

**Reagents and molecular cloning procedures.** Restriction enzymes, ligase, reverse transcriptase and T4 DNA polymerase were purchased from commercial sources and were used according to the suppliers’ recommendations. [α-32P]dCTP (300 Ci mmol⁻¹; 11-1 TBq mmol⁻¹) and [γ-32P]ATP (5000 Ci mmol⁻¹; 185 TBq mmmol⁻¹) were purchased from Amersham.

Chromosomal DNA from L. lactis was extracted as previously described (Lenehouts et al., 1989, 1990). Minipreparation of plasmid DNA from E. coli was done either by the alkaline lysis method of Birnboim & Doly (1979) or (for sequencing purposes) by using the QiAprep Spin Plasmid Miniprep kit (Qiagen). DNA restriction enzyme fragments were isolated from agarose gels using the Qiagen Gel Extraction kit as recommended by the manufacturer. PCR fragments were purified using the Boehringer High Pure PCR Purification kit (Roche Diagnostics).

Electrotransformation of E. coli was accomplished using the Bio-Rad Gene Pulser apparatus according to the manufacturer’s instructions. Preparation and transformation of competent L. lactis cells were performed as described by De Vos et al. (1989). Southern transfer and hybridizations were all performed according to standard procedures (Sambrook et al., 1989) using the ECL kit (Amersham).

**PCR and inverse PCR.** PCR reagents (Tag polymerase, Expand DNA polymerase and dNTPs) were purchased from Roche Diagnostics and used according to the manufacturer’s instructions. Preparation of competent L. lactis cells were performed as described by De Vos et al. (1989). Southern transfer and hybridizations were all performed according to standard procedures (Sambrook et al., 1989) using the ECL kit (Amersham).

Template DNA for inverse PCR was prepared essentially as described by Ochman et al. (1990) with the following modifications. DNA restriction fragments were ligated under conditions that favoured the formation of monomeric circles (DNA concentrations ranging from 0.1 µg to 0.5 µg in a total volume of 50 µl were ligated for 16 h at 15 °C). The ligated DNA mixture was phenol-extracted, precipitated and subse-
Two-component systems in *Lactococcus lactis*

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

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Relevant properties</th>
<th>Source or reference</th>
</tr>
</thead>
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<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em></td>
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<tr>
<td>XL-1 Blue</td>
<td><em>recA1 endA1 gyrA96 thi-1 bsdR17 supE44 relA1 lac [F' proAB lacI</em>ZΔM15 Tn10(Tc'r)]*</td>
<td>Stratagene</td>
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<tr>
<td><em>L. lactis</em></td>
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<td></td>
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<tr>
<td>MG1363</td>
<td>Plasmid-free derivative of <em>L. lactis</em> subsp. cremoris NCDO 712</td>
<td>Gasson (1983)</td>
</tr>
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<td>MGpAK80-B</td>
<td>Em' derivative of MG1363 harbouring pAK80 containing 248 bp DNA fragment</td>
<td>This study</td>
</tr>
<tr>
<td>MGpAK80-D</td>
<td>Em' derivative of MG1363 harbouring pAK80 containing 297 bp DNA fragment</td>
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<td>Em' derivative of MG1363 harbouring pAK80 containing 268 bp DNA fragment</td>
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<td>MGpAK80-F</td>
<td>Em' derivative of MG1363 harbouring pAK80 containing 259 bp DNA fragment</td>
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<td>MGKinA</td>
<td>Em' derivative of MG1363 with integrated pRV300 containing internal 800 bp fragment of <em>llKinA</em></td>
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<td><strong>Plasmids</strong></td>
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<td>pBluescript SK(−)</td>
<td>Ap', αlacZ</td>
<td>Stratagene</td>
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<td>pRV300</td>
<td>Ap' Em'</td>
<td>Leloup <em>et al.</em> (1997)</td>
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<td>pAK80</td>
<td>Promoter selection vector containing promoterless <em>lacL</em> and <em>lacM</em> genes</td>
<td>Israelsen <em>et al.</em> (1995)</td>
</tr>
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<td>pLlrF</td>
<td>Ap', 4.2 kb fragment cloned in pBS II SK(−)</td>
<td>This study</td>
</tr>
</tbody>
</table>

Subsequently used as template for inverse PCR, which was performed using a Boehringer ExpandTM Long Template PCR system as recommended by the manufacturer.

**Nucleotide sequence analysis.** Nucleotide sequence determination was performed on an ABI 373A automatic sequencer using the Dye Terminator Sequencing kit (Applied Biosystems). Sequence data assembly and analysis was performed using DNASTAR software. Sequence comparisons and database searches were performed using non-redundant sequences present at the NCBI internet site (http://www.ncbi.nlm.nih.gov/) using TBLASTN and BLASTP programs (Altschul *et al*., 1997). Sequence alignments were performed using the CLUSTAL method of the MEGALIGN program of the DNASTAR software package. Protein analysis was performed by Kyte–Doolittle hydrophilicity plots of the PROTEAN program of the DNASTAR software package.

**Construction of promoter fusions.** DNA fragments containing presumptive promoters for the 2CSs were amplified by PCR using *L. lactis* MG1363 chromosomal DNA as template, ligated to the *BglII* and *PstI* restriction sites of the promoter probe vector pAK80 (Israelsen *et al*., 1995) and introduced into *E. coli* XL-1 Blue by electrotransformation. The expected
structure of the recombinant plasmids was confirmed by restriction mapping, PCR and sequencing of the cloned fragment, prior to their introduction into L. lactis MG1363 by electroransformation and subsequent selection on M17 glucose agar plates containing erythromycin (5 µg ml⁻¹) and X-Gal (40 µg ml⁻¹). Northern blot and primer extension analysis. Total RNA was isolated from L. lactis by the Macaloid method described by Kuipers et al. (1993). For Northern blot analysis, RNA was glyoxylated, separated by agarose gel (1%) electrophoresis, blotted and hybridized as described previously (Van Rooijen & de Vos, 1990). Hybridization probes derived from each 2CS [internal 300 bp fragments of each RR-encoding gene cloned in pBluescript SK(−)] were radiolabelled with [α-32P]dCTP by random priming (Roche Diagnostics). Primer extension was performed by annealing 10 pmol 5^-end γ-32P-labelled synthetic 18-mer oligonucleotides to 50 µg of RNA as described by Pujic et al. (1998). Sequence ladders were produced using the same primers as the primer extension product with the aid of the Sequenase sequencing kit (version 2.0) (US Biochemical).

Construction of RR and HPK mutants. Internal 500 and 800 bp fragments of the six identified RR and HPK genes, respectively, were amplified by PCR using L. lactis MG1363 chromosomal DNA as template. DNA fragments to be amplified were chosen so that after insertion into the chromosome the HPK- and RR-encoding genes would be interrupted, and in the protein products the conserved histidine/aspartate residues, which are the sites of phosphorylation, would be separated from their respective C-terminal transmitter/effect domains. The amplified fragments were cloned in the integration insertion vector pRV300 (Leloup et al., 1997) and introduced into competent L. lactis MG1363 cells by electroransformation, with subsequent selection on M17 glucose agar plates containing erythromycin (5 µg ml⁻¹). The colonies arising on this plate were suspected to have integrated pRV300 derivatives into their chromosome by a Campbell-like mechanism as plasmid pRV300 is incapable of replication in L. lactis. This assumption was confirmed by PCR mapping and Southern hybridization analyses using chromosomal DNA prepared from these transformants.

Sugar metabolism profiles. Sugar metabolism profiles were determined using API 20 Strep as recommended by the manufacturer (BioMérieux). The resulting fermentation patterns were inspected following incubation at 30 °C for 4 and 24 h. Fermentation of carbohydrates was detected by acid production and a change in colour of the pH indicator.

Determination of acid stress resistance. Overnight cultures were diluted 100-fold in TYG broth and grown at 30 °C to early-exponential-growth phase (OD₆₀₀ ~ 0.3). These cells were used for acid stress resistance experiments using two distinct procedures: (1) cells were spread-plated on modified GM17 agar (β-glycerophosphate absent and pH adjusted to a value of 5.5 with acetic acid after sterilization) and incubated at 30 °C overnight, after which colony numbers were evaluated; and (2) (according to O’Sullivan, 1996) cells were harvested, resuspended in TYG broth, which had been adjusted to pH 4.0 with acetic acid, incubated at 30 °C and spread-plated on TYG agar at hourly intervals for 3 h. The plates were incubated at 30 °C overnight, after which the number of colony-forming units was determined.

Determination of osmotic and oxidative stress resistance. Overnight cultures were diluted 100-fold in TYG broth and grown at 30 °C to early-exponential-growth phase (OD₆₀₀ ~ 0.3). They were then harvested and resuspended in TYG broth containing 20% NaCl or 4 mM H₂O₂ to assess osmotic or oxidative stress resistance, respectively. Cultures were subsequently incubated at 30 °C for 8 h and 20 min, respectively, and serial dilutions spread-plated at appropriate time intervals. Plates were incubated at 30 °C overnight, after which the stress resistances were determined by colony-counting (O’Sullivan, 1996).

Phosphatase activity and β-galactosidase assays. Qualitative phosphatase activity was determined by streaking cultures on GM17 agar containing X-P (40 µg ml⁻¹) and incubating overnight at 30 °C. A blue colour indicated a phosphatase-positive culture. β-Galactosidase activity was determined as described by Israelsen et al. (1995).

RESULTS
Identification and sequencing of putative RR-encoding genes from L. lactis by chromosome walking

In a previous paper, complementation of HPK-deficient E. coli mutants allowed the identification of five putative HPK-encoding genes in L. lactis MG1363 (O’Connell-Motherway et al., 1997). The genes encoding the HPK and RR of a particular 2CS are frequently organized as an operon (Parkinson, 1993; Hoch & Silhavy, 1995), and therefore it was expected that sequencing the regions surrounding the five HPK-specifying genes would reveal, at least in some cases, the gene encoding the presumptive cognate RR.

The HPK-encoding genes were used as probes to determine the restriction map of their surrounding regions by Southern hybridization (data not shown). Based on the five HPK nucleotide sequences, primers were designed to amplify DNA regions neighbouring each HPK gene by inverse PCR. Sequence analysis of the PCR products allowed the identification of a putative RR-specifying gene for each of the putative HPK-encoding genes. These genes were designated IlkR–E (where the capital letter is identical to that of the corresponding HPK-encoding genes IlkR–D) and were located either upstream (in the case of IlkR, IlkC and IlkE) or downstream (in the case of IlkD) of the putative HPK-encoding gene (Fig. 1).

Cloning of putative RR-encoding genes from L. lactis by PCR amplification

Receiver modules of RR proteins show a high degree of conservation (Pao & Saier, 1995; Volz, 1995). In a previous study, it was verified that a single PCR performed with a set of two degenerate oligonucleotide primers (DD and K primers) complementary to the well-conserved DD and K boxes present at each end of the receiver module domain (Parkinson, 1993) allowed rapid and accurate isolation of internal fragments of genes encoding putative RRs from a wide range of bacteria (Morel-Deville et al., 1997, 1998)

This strategy was applied to isolate (internal fragments of) RR-encoding genes from L. lactis MG1363. A unique and intense band of the expected size (approx. 290 bp)
Two-component systems in *Lactococcus lactis*

**System A**

- **IlcsAorf1**
  - **IlrA**
  - **IlkinA**
  - **IlcsAorf2**
  - **IlcsAorf3**
  - ~2.5 kb transcript

**System B**

- **IlcsBorf1**
  - **IlrB**
  - **IlkinB**
  - **IlcsBorf2**
  - **IlcsBorf3**
  - **IlcsBorf4**

**System C**

- **IlcsCorf1**
  - **IlrC**
  - **IlkinC**
  - **IlcsCorf2**
  - ~3.0 kb transcript
  - **groEL**

**System D**

- **IlcsDorf1**
  - **IlkinD**
  - **IlrD**
  - **IS981**

**System E**

- **IlcsEorf1**
  - **IlrE**
  - **IlcsEorf2**
  - **IlkinEorf1**
  - **IlkinEorf2**
  - **IlcsEorf4**

**System F**

- **IlcsForf1**
  - **IlrF**
  - **IlkinF**
  - **IlcsForf4**

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**Fig. 1.** Schematic representation of the six 2CSs and each of their surrounding ORFs. Arrows represent each ORF with the gene name positioned above. The positions of the two promoters identified by Northern analysis and primer extension analysis are indicated by `P` and the extent of each of the transcripts is indicated by a narrow arrow. DNA fragments cloned in pAK80 are represented by a hatched line and the presence of a promoter indicated by `P`. The positions of putative terminator structures are indicated by `q`.

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was generated (data not shown). The assumed mixture of DNA fragments was purified, cloned into the unique EcoRI/XbaI sites of the pBluescript II SK(−) vector and sequenced on both strands. Analysis of the nucleotide sequence of 48 independent clones revealed that they corresponded to four different DNA sequences. Each of the four sequences of the coding strand contained one uninterrupted ORF. Further inspection of the four DNA sequences revealed that three fragments were internal segments of the putative RR-encoding genes designated *IlrA*, *IlrC* and *IlrE* described above. Analysis of the deduced amino acid sequence of the ORF contained within the fourth segment also showed significant similarity to RR receiver modules in the sequence databases (data not shown). These results provided strong evidence that this DNA segment amplified by the primer set originated from a RR gene of *L. lactis* MG1363. Extensive screening of 600 independent clones by colony hybridization with the four cloned DNA segments as probes did not allow identification of other putative RR-encoding genes in MG1363. This may be due to the primer design, which may have to be adapted for *L. lactis* (Morel-Deville *et al.*, 1997), or to the possible paucity of 2CSs in this bacterium.

The entire sequence of the newly identified putative RR-encoding gene, designated *IlrF*, was determined by cloning a 4–2 kb XbaI chromosomal DNA fragment from *L. lactis* MG1363 which hybridized with the fourth DNA fragment described above in pBluescript II SK(+) to produce plasmid pLlrF. This clone contained the complete coding region (*IlrF*) of this putative RR protein as well as two upstream ORFs. Inverse PCR allowed amplification of DNA downstream of *IlrF*. Subsequent chromosome walking and DNA sequence analysis revealed the presence of a putative HPK-encoding gene, designated *IlkinF*, immediately downstream of *IlrF*. The deduced protein product of *IlkinF* is significantly similar to *IlkinA*, *IlkinB*, *IlkinC* and *IlkinEorf1* (O’Connell-Motherway *et al.*, 1997) and to the Env/PhoR family of HPKs, possessing the four conserved histidine kinase motifs: the autophosphorylation site (H box), at which a conserved histidine is phosphorylated upon signal detection; the phenylalanine/aspartate (F/D) and glycine (G) boxes, which are thought to be involved in nucleotide binding; and the asparagine (N) box, which is of unknown function. Two hydrophobic membrane-spanning segments were identified, suggesting that this protein is composed of a non-conserved N-terminal
membrane-associated signal input (sensor) domain and a conserved C-terminal intracytoplasmic domain required for subsequent signal transmission to the regulatory partner through phosphorylation (Stock et al., 1995) (Fig. 2).

**Structural features of LlrA–F**

LlrA, LlrB, LlrC, LlrE and LlrF show extensive similarities to each other throughout their entire effector domain and all possess the conserved amino acid residues D1, DD and K and the succession of β-strand and α-helix segments (Pao & Saier, 1995; Mizuno & Tanaka, 1997) (Fig. 3a). In addition, a non-canonical helix–turn–helix DNA-binding motif typical of the OmpR subfamily of RRs is obvious at the C terminus of these proteins (Mizuno & Tanaka, 1997). It appears therefore that these five RR proteins belong to the so-called OmpR subfamily of bacterial RRs, which also includes PhoB, VirG, ArcA, CreB and KdpE (Parkinson & Kofoid, 1992; Pao & Saier, 1995).

LlrD possesses a slightly smaller effector domain. Sequence comparisons indicated that this domain aligns with putative DNA-binding domains found in class III RRs (Pao & Saier, 1995; Baikalov et al., 1996) (Fig. 3b). These findings indicate that LlrD may be a DNA-binding regulatory protein belonging to the so-called NarL subfamily of bacterial RR proteins (Parkinson & Kofoid, 1992; Pao & Saier, 1995; Baikalov et al., 1996).

**Identification of genes surrounding each putative 2CS by chromosome walking**

Sequencing of the DNA regions adjacent to the six 2CSs allowed the identification of a number of neighbouring ORFs. These are summarized in Table 2 together with their similarities to known protein products. Their positions and orientations are schematically depicted in Fig. 1.

**Northern blotting and transcriptional start site mapping**

Transcription of the 2CS genes was analysed by Northern hybridization using RNA isolated from *L. lactis* grown in M17 broth containing 0–5% glucose at optical densities of 0–12, 0–6, 1–2 and 1–5. DNA probes derived from each 2CS [internal 300 bp fragments of each RR-encoding gene cloned in pBluescript SK(−)] were used to determine the size and timing of the transcripts. Under the conditions used, transcripts were detected only for systems A and C, and the lengths of these were approximately 2–5 kb and 3 kb, respectively. Systems A and C appear to be constitutively expressed as a single transcript, which did not vary in intensity, was observed on Northern blots performed from RNA isolated at various stages of growth (data not shown). The transcription start site for each system was subsequently identified by primer extension analysis. Upstream of these transcription start sites, vegetative promoters were
Two-component systems in *Lactococcus lactis* (a)

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![Multiple sequence alignment of LlrA, LlrB, LlrC, LlrE and LlrF with *E. coli* PhoB (a) and of LlrD with *E. coli* NarL (b).](image)

Amino acids are boxed if five or six (a) or both (b) the proteins contain identical residues at corresponding positions. Conserved motifs present in RRs designated DD, D and K are indicated by lines below the corresponding sequences. The succession of β-strand and α-helix segments is indicated by arrows and unshaded boxes, respectively, above the appropriate amino acids. Asterisks mark loop regions adjacent to the phosphorylation site. The secondary structure of the C-terminal DNA-binding domain of OmpR is depicted schematically above the multiple alignment in (a) with black arrows representing the β-strands, hatched boxes the α-helices and lines representing the loop regions.

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identified consisting of a consensus −10 hexanucleotide TATAAT and a −35 hexanucleotide which deviated somewhat from the consensus sequence TTGACA (Van de Guchte *et al.*, 1992; Jensen & Hammer, 1998) (Fig. 4a, b). Inspection of the sequence 2–5 or 3–0 kb downstream from the transcriptional start points of systems A and C, respectively, allowed the identification of rho-independent transcription terminator structures for each of these systems at positions which were consistent with the expected transcriptional end points (Fig. 1).

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**Characterization of β-galactosidase promoter fusions**

Transcription of the remaining four 2CS genes was analysed by constructing β-galactosidase promoter fusions using the promoter probe vector pAK80. Since
### Table 2. ORFs identified adjacent to *L. lactis* 2CS

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<tr>
<th>ORF</th>
<th>Size (aa)</th>
<th>Similar protein</th>
<th>Function</th>
<th>e-value</th>
<th>Similarity (%)</th>
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<td>LltcsAorf1</td>
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<td><em>B. subtilis</em> YlbN</td>
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<td>4e-13</td>
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<td>38</td>
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<tr>
<td>LltcsAorf3</td>
<td>113</td>
<td><em>B. subtilis</em> NrgB</td>
<td>Signals cellular nitrogen status</td>
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<td>55</td>
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<tr>
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<td><em>Streptococcus mutans</em> YlxM</td>
<td>Putative nutritional and stress response protein</td>
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<td>72</td>
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<tr>
<td>LltcsBorf2</td>
<td>146</td>
<td>No similarity in databases</td>
<td></td>
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<tr>
<td>LltcsBorf3</td>
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<td><em>B. subtilis</em> YvoA</td>
<td>Transcriptional regulator (Gntr family)</td>
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<td>32</td>
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<td>LltcsBorf4</td>
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<td><em>L. lactis</em> Exp3</td>
<td>Extracellular protein 3</td>
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<td>LltcsCorf1</td>
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<td><em>B. subtilis</em> thymidylate kinase</td>
<td>Phosphorylation of dTMP to form dTDP in both de novo and salvage pathways of dTTP synthesis</td>
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<td>LltcsDorf1</td>
<td>120</td>
<td><em>L. lactis</em> IS981*</td>
<td>Insertion sequence element</td>
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<td>100</td>
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<td>LltcsEorf1*</td>
<td>144</td>
<td><em>B. subtilis</em> CsbB</td>
<td>Glycosyl transferase involved in general stress response; belongs to DPM1 family of glycosyl transferases</td>
<td>3e-17</td>
<td>32</td>
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<td>LltcsEorf2</td>
<td>171</td>
<td>No similarity in databases</td>
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<td>LltcsEorf3*</td>
<td>59</td>
<td><em>B. subtilis</em> aminotransferase</td>
<td>L-Glutamine-d-fructose-6-phosphate amidotransferase</td>
<td>6e-11</td>
<td>60</td>
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<td>LltcsForf1</td>
<td>595</td>
<td><em>Lactobacillus sanfranciscensis</em> MalP</td>
<td>Malrose phosphorylase</td>
<td>2e-66</td>
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<td><em>Lactobacillus sanfranciscensis</em> MalR</td>
<td>Malrose operon transcriptional repressor</td>
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<td>31</td>
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<tr>
<td>LltcsForf3</td>
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<td><em>B. subtilis</em> DXP synthase</td>
<td>1-Deoxyxylulose-5-phosphate synthase</td>
<td>8e-40</td>
<td>50</td>
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* Complete DNA sequence not determined in this study.

primer extension analysis had shown that the promoters of systems A and C are located within non-coding regions immediately upstream of *llrA* and *llrC*, similar non-coding regions upstream of *llrB*, *lltcsDorf1* (this is the first non-coding region upstream of *llrD*), *llrE* and *llrF* (see Fig. 1) were amplified by PCR and cloned into pAK80. The resulting plasmid constructs were introduced into *L. lactis* MG1363 by electrottransformation and plated on GM17 agar containing erythromycin (5 μg ml⁻¹) and X-Gal (40 μg ml⁻¹). All transformants produced colonies exhibiting varying degrees of blue colour, thus indicating the presence of an active promoter within each cloned DNA fragment. The strains harbouring the pAK80 constructs were designated MGpAK80-B to MGpAK80-F (where MGpAK80 indicates MG1363 harbouring the plasmid pAK80 and the capital letter denotes the 2CS from which the promoter-containing fragment had originated).

Growth profiles were determined in GM17, TYG broth and minimal medium at 30 °C and β-galactosidase activity was measured as a function of growth. In all media MGpAK80-B and MGpAK80-F exhibited a sharp increase in β-galactosidase activity which started at the mid-exponential phase of growth and continued to increase into stationary phase (Fig. 5). MGpAK80-D and MGpAK80-E also exhibited inducible β-galactosidase activities but in these two cases the induction level appeared to be much lower. Strain MGpAK80-E displayed a β-galactosidase activity profile similar to that of MGpAK80-B and MGpAK80-F, whilst in the case of MGpAK80-D activity started to increase in late-exponential phase and this increase continued into stationary phase (Fig. 5).

**Characterization of HPK and RR mutants**

To obtain information as to the possible regulatory functions of the putative 2CSs, insertion mutants of *L. lactis* MG1363 were constructed by gene disruption using derivatives of the non-replicative delivery vector pRV300 (Leloup et al., 1997), which carried central regions of each HPK and RR gene in such a way that it was assumed that in each case sequences encoding conserved catalytic domains had been interrupted by the insertion event (for details, see Methods). These constructs were confirmed by Southern hybridization analysis (data not shown). Several attempts were made to construct insertion mutants of *llkinD* and *llkinF*; however, these were unsuccessful, possibly because these mutations are lethal to the lactococcal cell. The mutated strains that were obtained were designated MGKinA, MGKinB, MGKinC, MGKinE or MGRRa–F (where the denotation ’Kin’ or ‘Rr’ in the strain name refers to the nature of the mutation, either in a HPK- or RR-encoding gene, respectively, and the capital letter A–F represents
the identity of the interrupted 2CS). Since the receiver domain and the effector domain coding sequences were dissociated in these mutants, these strains are likely to represent functional null mutants in each of the genes. The ability of these mutant strains to grow in M17 broth containing 0-5% glucose was monitored. MGKinA and MGRrA were clearly impaired in growth as compared to other mutant strains or to the wild-type strain (data not shown). MGKinC was also impaired in growth and it exhibited an additional clumping phenotype, which was not observed for MGRrC. Other mutants behaved like the wild-type cells at 30 °C. In view of the fact that 2CSs are often involved in environmental sensing in bacteria, the cellular response of these mutant strains to various stress conditions was examined. A number of tests were performed in an attempt to disclose the regulatory pathway in which each 2CS could be involved. For a number of tests performed (antibiotic resistance, heavy metal resistance and bacteriophage sensitivity experiments) no differences were observed between the wild-type, MG1363, and the mutant cultures, and for such experiments the data are not shown.

API profiles

API 20 Strep profiles were performed to determine if the mutants had been altered with respect to sugar metabolism. No differences in sugar metabolism were observed; however, it was observed that MGKinA and MGRrA were arginine-deaminase-negative whilst the parent strain, MG1363, and the other mutant strains gave a positive reaction. This observation was confirmed by growing MGKinA and MGRrA in Niven’s arginine broth and testing for the production of ammonia from arginine using Nessler’s reagent. Upon addition of Nessler’s reagent, a brown precipitate was observed for MG1363, indicating a positive arginine deaminase reaction, but was not observed for MGKinA and was observed to a lesser extent for MGRrA. This result indicates that system A is involved in regulation of arginine metabolism in the lactococcal cell.

Acid stress

Two methods were used to assess the effect of acid stress on the MG1363 parent and the HPK and RR mutant strains (see Methods). Using the first procedure, it was

Fig. 4. Determination by primer extension analysis of the transcription start site of 2CS A (a) and 2CS C (b). Lane PE shows the primer extension product from total RNA from *L. lactis*. Sequence ladders were run in parallel and were produced using the same primers as the primer extension product. The nucleotide sequences of the promoter regions (non-transcribed strand) of 2CS A and 2CS C including the 5'-end of *llrA* (a) and *llrC* (b) are shown on the right. Potential promoter elements (−10 and −35) and the ribosome-binding site (SD) are indicated in bold. The positions of the transcription start sites (TSS) are in bold and marked with an asterix above their nucleotides. The ‘M’ represents the methionine residue which is the first amino acid of the RR proteins. Arrows indicate the precise start point and direction of the primers used.
found that MGKinA and MGRrA were 10-fold more acid-resistant, whilst MGKinB and MGRrB were 100-fold more acid-resistant, compared to the parent strain, MG1363. However, it should be noted that the colonies of MGKinB and MGRrB strains were pinpoint-sized and only became visible after 72 h incubation when cultures were plated on pH-adjusted, unbuffered GM17 agar. This acid resistance was not observed using the second procedure whereby parent and mutant cultures grown in TYG broth to early-exponential phase were harvested and resuspended in TYG broth which had been adjusted to pH 4.0 with acetic acid and incubated at 30 °C. Serial dilutions were plated at time zero and subsequently at hourly intervals to obtain plate counts (Table 3). After 3 h incubation, 0.4% of the original MG1363 cells were viable. MGKinB and MGRrB displayed slight acid resistance, having 1% survival after 3 h. An acid-sensitive phenotype was also observed for MGKinC and MGRrC, with only 0.008 and 0.000002% survival, respectively, after 3 h at pH 4.0. Strains MGKinA and MGRrA exhibited a more acid-sensitive phenotype, with only 0.002 and <0.1% survival.

Table 3. Effect of acid stress (TYG broth, pH adjusted to a value of 4.0) on L. lactis MG1363 and HPK and RR insertion mutants

<table>
<thead>
<tr>
<th>Culture</th>
<th>Length of exposure to acid stress (h):</th>
<th>% Survival after 3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>MG1363</td>
<td>1.4 (±0.56) × 10⁶</td>
<td>5.7 (±0.31) × 10⁶</td>
</tr>
<tr>
<td>MGKinA</td>
<td>3.5 (±0.18) × 10⁷</td>
<td>7.3 (±0.1) × 10⁶</td>
</tr>
<tr>
<td>MGRrA</td>
<td>5.5 (±0.20) × 10⁷</td>
<td>&lt;10⁷</td>
</tr>
<tr>
<td>MGKinB</td>
<td>6.7 (±0.50) × 10⁷</td>
<td>6.8 (±0.42) × 10⁷</td>
</tr>
<tr>
<td>MGRrB</td>
<td>6.0 (±0.86) × 10⁷</td>
<td>6.5 (±0.40) × 10⁷</td>
</tr>
<tr>
<td>MGKinC</td>
<td>1.5 (±0.20) × 10⁷</td>
<td>1.2 (±0.30) × 10³</td>
</tr>
<tr>
<td>MGRrC</td>
<td>3.5 (±0.30) × 10⁷</td>
<td>6.0 (±0.4) × 10³</td>
</tr>
</tbody>
</table>

Fig. 5. Growth profiles (□) and β-galactosidase activities (●) of MGpAK80-B, MGpAK80-D, MGpAK80-E and MGpAK80-F in TYG broth at 30 °C. As a control the β-galactosidase activity of L. lactis harbouring pAK80 was measured as a function of growth in TYG broth. At the times indicated samples were withdrawn to determine β-galactosidase activity.
respectively, surviving the 3 h incubation at pH 4.0. The acid-sensitive phenotype observed for mutants of system A combined with the arginine deaminase-negative phenotype described above suggests that this 2CS may be involved in modulating the internal pH of the lactococcal cell and counteracting acid stress through arginine metabolism and its concomitant production of ammonia.

Salt stress
Salt stress experiments were performed in TYG broth. Early-exponential-phase cultures were harvested and resuspended in TYG broth containing 20% NaCl and incubated at 30 °C for 8 h, plating serial dilutions at 2 h intervals. After 8 h incubation, 5 1% of MG1363 cells were viable. Of the mutant strains tested, only MGGrD exhibited greater salt sensitivity as compared to the parent culture MG1363 with 1% of MGGrD surviving after 8 h (data not shown). From this result it would appear that system D may be involved in responding to salt or osmotic stress.

Oxidative stress
Early-exponential-phase cultures grown in TYG broth were harvested and resuspended in TYG broth containing 4 mM H2O2 and incubated at 30 °C. Samples were taken at time zero and after 10 and 20 min and serial dilutions plated. After 20 min incubation, 70% of the MG1363 cells remained viable. Of the mutant strains tested, only MGGrF exhibited greater H2O2 sensitivity as compared to the parent culture MG1363 with just 9% of MGGrF cells remaining viable after 20 min (data not shown). This observation suggests that system F may be involved in the response of L. lactis to oxidative stress.

Phosphatase activity
Qualitative phosphatase assays of MG1363 and the insertion mutant strains were established by streaking overnight cultures on GM17 agar containing an indicator of phosphatase activity, X-P, and incubating at 30 °C overnight. Phosphatase activity was observed for MG1363, indicated by blue-coloured colonies. All mutant colonies displayed a blue colony morphology similar to the wild-type, MG1363, except MGKnE, which produced colonies remaining completely white indicating an absence of phosphatase activity, and MGReE, which formed pale blue colonies, thus exhibiting very weak phosphatase activity.

DISCUSSION
This report describes the identification of six putative 2CSs from L. lactis MG1363, each consisting of a HPK and RR-encoding gene pair. We previously reported the identification of five putative HPKs from L. lactis by complementation of sensor-negative E. coli mutants (O’Connell-Motherway et al., 1997). Morel-Deville et al. (1997) employed an alternative approach to identify 2CSs, which involved amplification of internal fragments of RR-encoding genes using degenerate oligonucleotides based on the conserved DD and K regions of RR. Using this method, internal fragments of four lactococcal RR-encoding genes were identified.

The genes encoding corresponding HPK and RR proteins of a specific 2CS are frequently organized in an operon. This appears to be valid for all six 2CSs described here. Putative RR genes (designated llrA–D) were identified upstream of llkinA, llkinB and llkinC, and downstream of llkinD. One RR-encoding gene, llrE, was identified upstream of llkinE but separated by a putative ORF of 513 bp. This putative ORF, designated llcsEorf2, bears no significant homology to any known sequence in the databases, but the deduced amino acid composition and hydrophilicity profiles suggest that the gene product is a membrane-associated protein. Three of the four RR-encoding fragments identified using a PCR strategy were shown to be internal fragments of llrA, llrC and llrE. The remaining DNA fragment appeared to be part of an as yet unidentified RR-encoding gene, which was designated llrf. The complete DNA sequence of llrf was determined and a putative HPK gene designated llkinF was identified downstream of llrf.

Analysis of the deduced protein products of the six putative lactococcal RR-encoding genes demonstrated that they all possess the conserved N-terminal motifs characteristic of other RRs. They all contain an N-terminal receiver module of approximately 110 amino acids, which harbours the three signature amino acids typical for RRs.

Five of the lactococcal RRs, i.e. llrA, llrB, llrC, llrE and llrF, exhibit extensive similarity to the OmpR family of RRs, not only in the receiver domain but also in the effector domain (Fig. 3a). llrD on the other hand appears to belong to the NarL subfamily of RRs (Fig. 3b). In both OmpR and NarL subfamilies, the effector domain is a DNA-binding module, allowing the RR to function as a transcription factor. Phosphorylation serves to control the ability of the RR to either bind to a target DNA sequence or to interact with specific components of the transcription machinery.

The deduced protein product of llkinF exhibits strong structural similarity to members of the EnvZ family of orthodox HPKs. As with the other lactococcal HPKs (O’Connell-Motherway et al., 1997), two hydrophobic membrane-spanning segments were identified in LkinF, suggesting that this protein is composed of a non-conserved N-terminal membrane-associated signal input (sensor) domain and a conserved C-terminal intracytoplasmic domain required for subsequent signal transmission to the regulatory partner through trans-phosphorylation. These features suggest that all six identified L. lactis 2CS pairs function as signal transduction proteins via a mechanism of phosphorylation and dephosphorylation similar to those described for EnvZ-OmpR, NarX and NarQ-NarL and are likely to be involved in linking environmental awareness to
physiological adaptation through transcriptional regulation.

Northern analysis performed using RNA isolated during various stages of growth in GM17 at 30 °C established that only two of the systems, systems A and C, are constitutively expressed. DNA regions containing promoter activity were identified upstream of the other 2CSs. Examination of these regions by β-galactosidase gene fusions suggests that expression of these 2CSs is under the control of transcriptional regulation and is induced either during mid-exponential phase (systems B, E and F) or at the very end of the exponential phase (system D). The effect of various environmental factors on the mutant strains of the 2CSs was also examined. Mutant strains were constructed by disruption of the HPK- and RR-encoding genes by plasmid insertion and examined under various growth conditions. Polar effects of plasmid insertion on the expression of downstream-located genes cannot be excluded, but since RR- and HPK-encoding genes frequently form operons with genes involved in the same signalling pathway, no function other than that controlled by the 2CS should have been affected. Characterization of the insertion mutants obtained revealed that each had phenotypic differences from the wild-type, L. lactis MG1363, which ranged from acid sensitivity/resistance, altered phosphatase activity, increased salt sensitivity and H2O2 sensitivity.

From this preliminary characterization, it can be inferred that the adjacent gene couples of L. lactis MG1363 identified during this study probably encode members of homologous families of signal transduction proteins. The results obtained also suggest that all six systems are functional since promoter activity could be detected for each by Northern analysis or constructing β-galactosidase fusions, whilst in all cases phenotypic differences between the wild-type and mutant strains were observed.

Experiments are in progress to construct RR deletion mutants to verify the phenotypes observed with the insertion mutants and also to find target genes for at least some of the lactococcal 2CSs.

ACKNOWLEDGEMENTS

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REFERENCES


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