Molecular characterization of the lactococcal plasmid pCIS3: natural stacking of specificity subunits of a type I restriction/modification system in a single lactococcal strain

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A 6·1 kb plasmid from the Lactococcus lactis subsp. cremoris strain UC509.9, named pCIS3, was found to mediate a restriction/modification (R/M) phenotype. Nucleotide sequence analysis of pCIS3 revealed the presence of an hsdS gene, typical of type I R/M systems. The presence of this plasmid resulted in a 104-fold reduction in the efficiency of plating (e.o.p.) of unmodified phage. In addition to the hsdS gene of pCIS3, two more hsdS genes were identified in strain UC509.9, one located on the chromosome downstream of a gene highly homologous to hsdM genes and a third on the smallest (4 kb) plasmid, named pCIS1. The replication region of pCIS3 was highly similar to that of a large family of lactococcal theta replicons. In addition, pCIS3 was found to encode a member of the CorA family of magnesium transporters.

Keywords: Lactococcus lactis, restriction, modification, magnesium transport, cobalt resistance

INTRODUCTION

Lactococcal strains used in dairy fermentations are under continuous challenge by bacteriophages and, as a consequence, many strains have acquired several natural phage defence mechanisms. These are generally classified into four groups: (i) adsorption inhibition, (ii) injection blocking, (iii) abortive infection (abi) and (iv) restriction/modification (R/M; for reviews see Garvey et al., 1995; Allison & Klaenhammer, 1998). The first two mechanisms prevent the phage from entering the cell whilst mechanisms from the third group act on phage components after it has entered the cell. Adsorption inhibition is believed to be a result of the production of extracellular polymers such as polysaccharides that mask the phage receptor sites or, indeed, the complete absence of receptors. True injection blocking mechanisms where phage is capable of attaching to the cell but is unable to inject its DNA have been observed (Forde et al., 1999; Garvey et al., 1996), but to date none has been characterized at the molecular level. Abi encompasses a broad range of phage defence mechanisms that prevent phage proliferation after infection. To date 17 different Abi mechanisms have been identified. Most of these appear to interact at the level of DNA and/or RNA metabolism (replication, transcription and translation). The fourth type of resistance mechanism, R/M systems, is common in lactococci. These act on unmodified phage DNA as soon as it enters the cell whilst host DNA is protected from restriction by methylation at the cognate recognition sequence.

R/M systems are classified into three groups, based on their molecular structure, sequence recognition, cleavage position and co-factor requirements. Representatives of all three groups have been found in lactococci. The most common are type II R/M systems. These generally consist of two separate enzymes, one responsible for restriction, the other for modification. They typically recognize a palindromic sequence of 4–8 nt and cut the DNA within this sequence. Several R/M systems have been found that resemble the type II systems, but cut the DNA outside the recognition sequence. These have been subdivided as type IIs systems. A lactococcal example of a type IIs R/M system is the LlaI system from the conjugative plasmid pTR2030 (O’Sullivan et al., 1995). This system also
differs from other type II R/M systems because the restriction activity is encoded by three genes rather than one. Several type II R/M systems have been identified in lactococcal strains. As is common for most phage resistance mechanisms, all of these, with the exception of the ScrFI R/M system (Davis et al., 1993), were found to be plasmid located. Type III R/M systems consist of two subunits (Mod and Res) that form one functional holoenzyme with both restriction and modification activity. The enzyme recognizes specific asymmetric sequences and cuts the DNA at a fixed distance, 25–27 bp to one side of the recognition site. Recently, the first example of a lactococcal type III R/M system was identified and characterized (Su et al., 1999).

The most complex R/M systems are those of type I R/M. These consist of three subunits, named Hsd for host specificity determinant, that can form one holoenzyme. One subunit, HsdM, is required for modification of the recognition sequence whilst the third, HsdR, is required for restriction activity. Methylation can occur only in the presence of HsdS and HsdM and does not require ATP. Restriction, however, requires the presence of all three subunits, as well as ATP, and occurs at non-specific sites that can be more than 1 kb from the recognition sequence (Yuan, 1981). HsdS subunits consist of two variable domains separated and flanked by highly conserved sequences. It was shown that the first variable domain specifies the 5' DNA recognition sequence whereas the second specifies the 3' DNA recognition sequence, with a spacing of 6–8 nt between both (Fuller-Pace & Murray, 1986). As a consequence of their structure, variable domains can be swapped between the DNA regions encoding HsdS subunits via crossing-over events, resulting in novel hsdS genes with altered specificities (Fuller-Pace & Murray, 1986). Type I R/M systems have recently been shown to be widespread in lactococci and they could well be the most abundant R/M systems present in this group of bacteria (Schouler et al., 1998a, b). Several of these systems are plasmid located and the subunits may be located on one plasmid or on different plasmids (Forde et al., 1999; Schouler et al., 1998b) whilst others are chromosomally located (Schouler et al., 1998b).

*Lactococcus lactis* subsp. cremoris UC509.9 is a prophage-cured derivative of strain UC509 which was originally isolated from a mixed starter culture (Arendt et al., 1994) and can be used as an indicator strain for the temperate phage Tuc2009. It harbours six cryptic plasmids, denoted pCIS1 to pCIS6. We found that phage propagated on a UC509.9 derivative which was cured of pCIS3 exhibited a 10\(^4\)-fold reduction in the e.o.p. when used for the infection of *L. lactis* UC509.9 in the presence of pCIS3. Here, the entire nucleotide sequence of pCIS3 is reported. It was found to encode an *hsdS* gene, typical of type I R/M systems. Two additional *hsdS* genes were identified, one located on the chromosome and the other located on a 4 kb plasmid. The presence of multiple *hsdS* genes in a single host provides great potential for genetic recombination between them with the consequential possibility of increasing phage resistance. Furthermore, an ORF was identified on pCIS3, which shows homology to a ubiquitous class of magnesium transporters, known as *corA* (Kehres et al., 1998).

**METHODS**

**Strains, plasmids and phage.** All strains, plasmids and phages used in this study are listed in Table 1. Plasmid pCIS3 was genetically marked with an erythromycin-resistance gene by replacing a *PstI–SmaI* fragment of pBluescript with a *PstI–SmaI* fragment from pGKV210 (van der Vossen et al., 1987), resulting in pCIS31.1. As a consequence of this manipulation, the *corA* gene was deleted.

**Media and growth conditions.** *Escherichia coli* strains were grown in Luria–Bertani medium (LB) at 37 °C (Sambrook et al., 1989), supplemented with 100 µg ampicillin ml\(^{-1}\) or 100 µg erythromycin ml\(^{-1}\) when appropriate. *L. lactis* strains were grown at 30 °C in GSB, a modified version of LSB which contains glucose instead of lactose (Arendt et al., 1994), or in M17 broth (Difco), supplemented with 5 µg erythromycin ml\(^{-1}\) when appropriate.

**DNA manipulations.** Plasmid DNA was isolated from *E. coli* and *L. lactis* by using the Concert rapid plasmid isolation kit (Gibco-BRL). For *L. lactis* this was preceded by a 30 min incubation at 37 °C in solution G1 in the presence of 2 mg lysozyme ml\(^{-1}\) and 10 µM mutanolysin ml\(^{-1}\) to obtain clear lysates. Restriction enzymes and T4 DNA ligase were purchased from Boehringer and used according to the suppliers’ instructions. Oligonucleotides were synthesized using an Applied Biosystems 391 DNA synthesizer. The CaCl\(_2\) transformation method was used for *E. coli* (Dagert & Ehrlich, 1979) whilst *L. lactis* was transformed by electrottransformation as described by Wells et al. (1993). DNA fragments generated by PCR were subcloned in pCR2.1-TOPO TA using the TOPO TA cloning system (Invitrogen) according to the manufacturer’s instructions.

**Propagation of Tuc2009.** Propagation of phage was performed by growing the indicator strain to an OD\(_{600}\) of approximately 0.2–0.3 at which point CaCl\(_2\) was added to a final concentration of 10 mM, prior to the addition of phage. Incubation was subsequently continued until lysis occurred.

**Bacteriophage assays.** Plaque assays were carried out as described by Lillehaug (1997). Briefly, M17 double-layer agar plates, containing 0.5% glucose, 0.5% glycine and 10 mM CaCl\(_2\), were prepared with 1% agar for the bottom layer and 0.4% agar for the top layer. Media were sterilized by boiling for 5 min in a microwave oven. Plates contained approximately 30 ml 1% agar and were overlaid with 2.5 ml 0.4% agar, supplemented with 100 µl indicator strain (OD\(_{600}\) = 0.6–0.8) and appropriate dilutions of freshly prepared phage lysates.

**Subcloning of pCIS3 and sequence analysis.** Plasmid pCIS3 was isolated from an agarose gel and subjected to digestion with a number of endonucleases. A single linear DNA fragment was obtained upon digestion with the endonucleases EcoRI, BamHI and PstI. The latter was used for cloning of the plasmid into the *PstI* site of pBluescript SK\(^+\), generating pBIS3. The plasmid was subcloned by ligation of a partial Sau3A digest with a BamHI digest of pBluescript SK\(^+\). The ligation mixture was used to transform *E. coli* XL-1 Blue and...
Multiple HsdS subunits in one lactococcal strain

Table 1. Strains, plasmids and phage

<table>
<thead>
<tr>
<th>Strain, plasmid or phage</th>
<th>Relevant features</th>
<th>Source/reference</th>
</tr>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. cremoris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UC509.9</td>
<td>Derivative of UC509 strain cured of prophage</td>
<td>Costello (1988)</td>
</tr>
<tr>
<td>UC509.93</td>
<td>UC509.9-derived strain, cured of pCIS3</td>
<td>This work</td>
</tr>
<tr>
<td>UC509.935</td>
<td>UC509.9-derived strain, cured of pCIS3 and pCIS5, Lac⁻</td>
<td>This work</td>
</tr>
<tr>
<td>IL1403</td>
<td>Plasmid-free strain, indicator strain for phage bIL66 and 952</td>
<td>Chopin <em>et al.</em> (1984)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>recA1 lac endA1 gyrA96 yhi hsdR17 supE44 relA1 (F proAB lac1 q lacZ ∆M15 Tn10)</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCIS3</td>
<td>6 kb plasmid from <em>L. lactis</em> UC509.9</td>
<td>This work</td>
</tr>
<tr>
<td>pBluescript SKII + pBl53</td>
<td>Amp’, lacZα complementation</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pGKV210</td>
<td>Em’, pWVO1-derived plasmid carrying the erythromycin-resistance marker of pE194</td>
<td>Van der Vossen <em>et al.</em> (1987)</td>
</tr>
<tr>
<td>pCIS31.1</td>
<td>Em’, pCIS3 carrying the erythromycin-resistance gene of pGKV210 on a PstI–SmaI fragment from pGKV210</td>
<td>This work</td>
</tr>
<tr>
<td>pFDi18</td>
<td>Cm’, pCIS05 (Hayes <em>et al.</em>, 1991) derived, carrying the nonsense suppressor supB</td>
<td>Dickely <em>et al.</em> (1995)</td>
</tr>
<tr>
<td><strong>Bacteriophages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tuc2009</td>
<td>Temperate phage, isolated from <em>L. lactis</em> UC509</td>
<td>Arendt <em>et al.</em> (1994)</td>
</tr>
<tr>
<td>952</td>
<td>Lytic phage for IL1403</td>
<td>University College, Cork collection</td>
</tr>
<tr>
<td>bIL66</td>
<td>Lytic phage for IL1403</td>
<td>Bidnenko <em>et al.</em> (1995)</td>
</tr>
</tbody>
</table>

Transformants were selected on plates containing X-Gal. Plasmid DNA from individual white colonies was isolated, checked for the presence of inserts and used for sequence analysis that was performed with an Applied Biosystems 373A automated DNA sequencer. Assembly of sequences was done with the seqman program of the dnaStar software package. Database searches were performed with the programs blastp, blastn and tblastn (Altschul *et al.*, 1997). Finally, oligonucleotides flanking the PstI site were used to generate a PCR fragment using pCIS3 as a template. This fragment was sequenced in order to confirm that the PstI site was unique and not closely linked to another PstI site.

**RESULTS**

Plasmid curing and identification of an R/M phenotype

The prophage-cured strain *L. lactis* UC509.9 carries a total of six plasmids, named pCIS1 to pCIS6 (Fig. 1, lane 1). Introduction of the pCIS05-derived plasmid pFDi18 (Dickely *et al.*, 1995), which carries the nonsense suppressor supB and confers chloramphenicol resistance on UC509.9, appeared to be associated with the loss of pCIS3. It was found that phage propagated on this strain, designated UC509.93(pFDi18), infected *L. lactis* UC509.9 with an e.o.p. of 10⁻⁴. Phage isolated from individual plaques on *L. lactis* UC509.9 gave equal plaque counts on both *L. lactis* UC509.9 and UC509.93(pFDi18) (results not shown). To show that this phenotype, typical for R/M systems, resulted from the loss of pCIS3 rather than the introduction of pFDi18, UC509.93(pFDi18) was cured of pFDi18 by continuous growth in the absence of antibiotic selection for over 100 generations. Samples were plated on GM17 and conducted using the ECL gene detection system (Amersham) as recommended by the manufacturer.
individual colonies were replica plated on chloramphenicol-containing plates to test for the loss of antibiotic resistance. Plasmid profiles of chloramphenicol-sensitive colonies showed that these had indeed lost pFDi18.

Continuous subculturing of this strain in the presence of glucose in some cases also resulted in the loss of another plasmid, pCIS5. This strain, denoted \textit{L. lactis} UC509.935 (Fig. 1, lane 2), was no longer capable of growing in media with lactose as the sole carbon source, indicating that pCIS5 encodes one or more lactose-metabolizing genes, a trait which is typically found to be linked to larger (>20 kb) plasmids (McKay, 1983).

As can be seen from Fig. 1, pCIS1 appears smaller when isolated from UC509.935 than when isolated from UC509.9. This is possibly due to some deletion event. Endonuclease treatment showed that pCIS1 from UC509.935 is approximately 100 bp smaller than pCIS1 isolated from UC509.9.

**DNA sequence analysis**

The entire nucleotide sequence of pCIS3 was determined. It consists of 6159 bp with a total G+C content of 35.9 mol%. Four ORFs could be identified (Fig. 2, Table 2). The first ORF, designated \textit{rep}, shows high similarity to a family of replication genes that are commonly found in lactococcal strains and encode the replication-initiation protein. The highest level of similarity was found to the \textit{rep} gene of pCI305. \textit{rep} is followed by an ORF of unknown function, designated \textit{orfX}. Directly downstream of \textit{orfX}, a third ORF is found, which shows high levels of homology to \textit{hsdS} genes of type I R/M systems. Alignment of the deduced amino acid sequence of this gene with the \textit{hsdS}-encoded proteins of other lactic acid bacteria clearly shows the conserved domains, separated by variable domains (Fig. 3a). The N-terminal constant region is repeated at the 3' end of the middle region, while the conserved C-terminal domain is repeated at the 5' end of the middle region. \textit{rep}, \textit{orfX} and \textit{hsdS} are organized in an operon-like structure with the last two codon sequences of \textit{rep} overlapping the first two of \textit{orfX} and the last three codon sequences of \textit{orfX} overlapping the first three of \textit{hsdS}. No putative promoter sequences could be identified directly upstream of \textit{orfX} and \textit{hsdS}. Each is, however, preceded by near-consensus ribosome-binding sites.

pCIS3 encodes a fourth ORF, designated \textit{corA}, whose gene products display significant similarity to a ubiquitous class of magnesium transporter proteins with affinity for cobalt and nickel.

**Natural stacking of \textit{hsdS} genes in \textit{L. lactis} UC509.9**

Recombination between the variable domains of different \textit{hsdS} genes in a single strain is likely to play a role in ensuring continued dynamics in phage defence. In addition, plasmids can be mobilized between strains and if the conjugated plasmid carrying an \textit{hsdS} gene is introduced into a strain that already carries a different \textit{hsdS} locus, the unique structure of these genes allows recombination to occur, resulting in novel \textit{hsdS} genes with altered specificities (Fuller-Pace \textit{et al.}, 1984). This also implies that one strain could carry two or more \textit{hsdS} genes. To determine if this is the case in \textit{L. lactis} UC509.9, two oligonucleotides were designed, based on
Table 2. Co-ordinates of identified ORFs on pCIS3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Co-ordinates</th>
<th>Homology to</th>
<th>E value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rep</td>
<td>503–1663</td>
<td>pCIS305 repA</td>
<td>0.0</td>
<td>Hayes et al. (1991)</td>
</tr>
<tr>
<td>orfX</td>
<td>1656–2288</td>
<td>pL7 orfX</td>
<td>3.1 × 10^{-7}</td>
<td>Segers et al. (1994)</td>
</tr>
<tr>
<td>hsdS</td>
<td>2276–3508</td>
<td>pL7 hsdS</td>
<td>4.4 × 10^{-17}</td>
<td>Schouler et al. (1998a)</td>
</tr>
<tr>
<td>corA</td>
<td>3932–4843</td>
<td>Methanococcus jannasch corA</td>
<td>2.1 × 10^{-8}</td>
<td>Bult et al. (1996)</td>
</tr>
</tbody>
</table>

Genes with the highest level of identity as found in the database are indicated as well as the level of identity which is given as the E value. The closer the E value is to 0, the higher the level of homology.

Fig. 3. (a) Alignment of the deduced amino acid sequence of pCIS3 with those of pCIS65st (GenBank accession no. AF034786), pL7 (AF013596), pL2614 (U90222), pND861 (AF034786), pL103 (AF013595), the chromosomal hsdS gene from L. lactis IL1403 (AF013165) and pNZ4000 (AF036485). The region corresponding to the conserved 5' end of each hsdS domain is underlined with a thin line. The region corresponding to the conserved 3' end of each hsdS domain is underlined with a thick line. Rightward pointing arrows indicate the relative positions of SC1. Leftward pointing arrows indicate the relative positions of SC2. (b) Alignment of deduced amino acid sequences of fragments that were obtained by PCR using primers SC1 and SC2, with total DNA of L. lactis UC509.935 as target. The locations of SC1 and SC2 are underlined. Obtained fragments were of the expected length and encoded an ORF in one of the frames over the full length of the determined sequence.
the conserved domains that flank the variable domains of hsdS genes (SC1, 5′-GATTGGGAAGAGCTTGGTTT-3′ and SC2, 5′-AATTCCGCGATGAAGA-3′). These were used for PCR amplification of total DNA isolated from UC509.935. A product of the expected size was obtained and subcloned into pCR2.1-TOPO TA. Eight individual colonies were checked for inserts and used for sequence analysis. Three variable domains, designated V3, V4 and V5, differing from those of pCIS3, could thus be identified, indicating the presence of at least two additional hsdS genes in this strain. The alignment of the protein products specified by V3, V4 and V5 is presented in Fig. 3(b). The combined PCR products were used for Southern analysis. This confirmed the presence of two additional hsdS genes, one of which was found to be chromosomally located, whilst the other was located on pCIS1, the smallest plasmid present in UC509.9 (approx. 4 kb; Fig. 4b, lane 4).

The hsdM subunit of UC509.9 is similar to hsdM of L. lactis IL1403

Schouler et al. (1998b) designed oligonucleotides (designated M3, M4, M5 and M6) that were based on conserved sequences within a number of hsdM genes.

Fig. 4. Southern analysis of total DNA from UC509.9 and UC509.93 either uncut (lanes 1 and 3, respectively) or digested with HindIII (lanes 2 and 4, respectively). X indicates the position of chromosomal DNA. (a) Probe DNA hsdM, obtained by PCR using oligonucleotides M5 and M6 with total genomic DNA of UC509.9 as template. (b) Probe DNA hsdS, obtained by PCR using oligonucleotides SC1 and SC2 with total genomic DNA of UC509.9 as template. The faint band at the bottom of lanes 2 and 4 in (b) corresponds to the upper of the bottom two bands in lanes 2 and 4 of (a). The signal for the uncut chromosomal DNA in (b) was not strong enough to be visible under the conditions employed. Bands at the top of lanes 2 and 3 in (a) result from DNA in the wells that failed to enter the gel. The additional bands in lanes 1 and 3 in (b) between 6 and 7 kb represent open circular plasmid DNA of pC11.

Table 3. Effect of the HsdS subunit of pCIS31.1 on the e.o.p. of phage Tuc2009 propagated on UC509.93, and phages 952 and bIL66 propagated on IL1403

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>Phage</th>
<th>Tuc2009</th>
<th>952</th>
<th>bIL66</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC509.9</td>
<td>2.3 × 10⁻⁴</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>UC509.93</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>UC509.93(pCIS31.1)</td>
<td>2.5 × 10⁻⁴</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>IL1403</td>
<td>NA</td>
<td>1</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>IL1403(pCIS31.1)</td>
<td>NA</td>
<td>1.4 × 10⁻³</td>
<td>0.9 × 10⁻³</td>
<td>NA</td>
</tr>
</tbody>
</table>

Identical oligonucleotides were used for PCR amplification experiments on total DNA from UC509.9 in order to verify the presence of an hsdM subunit. A fragment of approximately 650 bp was obtained with oligonucleotides M5 and M6. The nucleotide sequence of this fragment was determined and shown to be nearly identical to the nucleotide sequence of a corresponding fragment of the hsdM gene of L. lactis IL1403 (nucleotide accession number AF013165). Southern hybridization experiments with this fragment showed that it was chromosomally located in UC509.9 (Fig. 4a, lanes 1 and 3).

To determine the orientation of the chromosomally encoded hsdS gene with respect to the hsdM gene, PCR reactions were performed with several combinations of the oligonucleotides M5, M6, SC1 and SC2. A product with a size of approximately 1 kb was obtained with the combination of M5 and SC2. All other combinations with the M and SC oligonucleotides failed to give a product. This indicates that the hsdS gene is located directly downstream of the hsdM gene in an orientation similar to that in L. lactis IL1403. The close proximity of hsdM to hsdS on the chromosome was confirmed by Southern hybridization where a common band of 1.2 kb was observed when either part of the hsdM gene or a combination of variable domains from all hsdS genes was used as a probe (Fig. 4a and 4b, lanes 2 and 4, respectively).

Effectiveness of HsdS subunits

The effect of the HsdS subunit of pCIS3 on the e.o.p. of phage Tuc2009 on UC509.9 and phage bIL66 and 952 on IL1403 was studied. For this purpose pCIS3 was genetically marked with the erythromycin-resistance gene of pGKV210 to create pCIS31.1 (see Methods). This plasmid was introduced into L. lactis strains UC509.93 and IL1403. Phage was then isolated from UC509.93 or IL1403 and used to infect both strains in the presence or absence of pCIS31.1 (Table 3). Whereas the presence of pCIS31.1 resulted in a 10⁴-fold decrease in the e.o.p. for Tuc2009 on UC509.93, this effect was markedly less (only a 10-fold decrease) for bIL66 and...
Multiple HsdS subunits in one lactococcal strain

Fig. 5. Nucleotide sequence and deduced amino acid sequence of CorA. Three transmembrane domains were predicted by using the membrane domain prediction program TopPred2 (von Heijne, 1992; http://www.embl-heidelberg.de/Cdoerks/Package5.html), and are underlined in the sequence.

952 on IL1403. These values for IL1403 were similar to those observed by Schouler et al. (1998b) with the hsdS genes from pIL7 and pIL261 in conjunction with phage bIL67.

e.o.p. values, comparable to those obtained with the hsdS genes from pCIS31.1 and pIL7, were obtained when the hsdS gene encoded by pCI65st (O’Sullivan et al., 1999) was used in UC509.93 and IL1403 (E. Stanley, unpublished results).

Absence of corA results in increased cobalt resistance

ORF4 of pCIS3 encodes a protein with similarity to CorA proteins identified in many prokaryotes (Kehres et al., 1998), where function as the dominant Mg²⁺-uptake system. ORF4 is similar in size to other corA genes and its deduced protein product contains three putative transmembrane domains (Fig. 5). To assess the function of corA, its effect on the cobalt tolerance of the host strain was determined. For this purpose, both UC509.93 and UC509.9 were streaked on plates with increasing amounts of CoCl₂. UC509.93 (lacking pCIS3) was capable of growing at concentrations up to 5 mM CoCl₂, whereas UC509.9 could only grow to a maximum of 1 mM CoCl₂ (Fig. 6). Introduction of pCIS31.1, from which corA was deleted (see Methods), into UC509.93 restored phage resistance, but failed to increase cobalt resistance.

DISCUSSION

While creating a nonsense suppressor strain of the lactococcal strain UC509.9 by supplying a suppressor gene on a plasmid, it became apparent that phage isolated from the nonsense suppressor strain showed a marked decrease in the e.o.p. when used to infect the original host. However, the e.o.p. of phage that was reisolated from this original host reverted to a value of 1–0, which is typical for R{M systems. Comparison of plasmid profiles showed that this R/M phenotype could be attributed to the presence or absence of a single 6–2 kb plasmid. Nucleotide sequence analysis of this plasmid, denoted pCIS3, revealed the presence of an hsdS gene of type I R/M systems. Type I R/M systems are widespread among prokaryotes, but have only recently been identified in lactococci (Schouler et al., 1998a). Using oligonucleotides based on conserved regions within hsdM genes (Schouler et al., 1998b), it was also possible to identify an hsdM gene, similar to that of IL1403, on the chromosome of UC509.9. Furthermore, by using oligonucleotides based on the conserved domains of hsdS genes in PCR amplification reactions, it was possible to demonstrate that at least two additional hsdS genes were located in L. lactis UC509.9. Southern analysis confirmed the presence of a total of three hsdS genes, one on the chromosome and two on small plasmids.

The presence of mobilization functions on lactococcal plasmids allows for transfer of plasmids (and the traits they encode) between different strains. When a plasmid that carries an hsdS gene is transferred to a strain that
already carries such a gene, crossover events can occur, resulting in novel HsdS subunits with altered specificities. It can be envisaged that it is advantageous for a strain to carry more than one copy of the $hsdS$ genes in order to achieve a high level of variation, resulting in increased phage resistance.

PCR analysis indicated that the chromosomally located $hsdS$ gene lies downstream of the $hsdM$ gene in an orientation similar to that found for IL1403 (Schouler et al., 1998b). Based on the typical organization of type I R/M systems found in lactococci to date, it can be expected that the $hsdR$ gene is located upstream of $hsdM$.

The effectiveness of two $hsdS$ genes was evaluated for strains UC509.9 and IL1403. The effect on the e.o.p. of the genes that were tested was significantly greater in the UC509.9 host than in IL1403. The values that were obtained with pCIS3 and pCle6st for IL1403 were similar to those observed by Schouler et al. (1998a) for pIL7. Since the $hsdM$ genes of IL1403 and UC509.9 are nearly identical, it can be speculated that this difference in effectiveness could be related to the activity of HsdR. It can not, however, be excluded that phage Tuc2009 has more recognition sites for the pCIS3 and pCle6st subunits than bIL66 and phage 952.

Three other genes were identified on pCIS3. The $rep$ gene, encoding the replication-initiation protein, is located on a large family of theta replicating plasmids that is very common in lactococci (Seegers et al., 1994). A 22 bp sequence, repeated three and a half times, preceding this gene is thought to be the major compatibility factor (Foley et al., 1996; Gravesen et al., 1997). Indeed, comparison of this sequence from pCIS3 with sequences in the nucleotide database revealed that this region was 100% identical to that of pCI305 (Hayes et al., 1991). This latter plasmid was used for the construction of pFDi18, which carries the $supB$ gene (Dickely et al., 1995), which explains why pCIS3 was lost from the strain when pFDi18 was introduced and provides further evidence for the role of the repeated fragment in compatibility.

The first codons of the second gene, named orfX, overlap the last codons of $rep$. Similar genes are found in most plasmids of this family. To date no function has been attributed to this gene.

The amino acid sequence of the fourth gene that could be identified showed similarity to a group of magnesium-transporter proteins, commonly indicated as CorA. These genes have been shown to be ubiquitous in eubacteria and to encode their dominant Mg$^{2+}$-uptake system (Smith & Maguire, 1998). Loss of the plasmid resulted in an increase in cobalt resistance, indicating it has a similar function in UC509.9. However, the absence of corA did not result in any other visible effects on growth under the conditions used. Possible explanations for this could be the presence of another corA gene on the chromosome or the presence of additional Mg$^{2+}$-transporter proteins. To our knowledge this is the first example of such a gene located on a plasmid in bacteria.
Multiple HsdS subunits in one lactococcal strain

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