

## Identification of *Lactococcus lactis* Genes Required for Bacteriophage Adsorption

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The aim of this work was to identify genes in *Lactococcus lactis* subsp. *lactis* IL1403 and *Lactococcus lactis* subsp. *cremoris* Wg2 important for adsorption of the 936-species phages bIL170 and  $\phi$ 645, respectively. Random insertional mutagenesis of the two *L. lactis* strains was carried out with the vector pGh9:ISS1, and integrants that were resistant to phage infection and showed reduced phage adsorption were selected. In *L. lactis* IL1403 integration was obtained in the *ycaG* and *rgpE* genes, whereas in *L. lactis* Wg2 integration was obtained in two genes homologous to *ybcC* and *ybcB* of *L. lactis* IL1403. *rgpE* and *ybcB* encode putative glycosyltransferases, whereas *ycaG* and *ybcC* encode putative membrane-spanning proteins with unknown functions. Interestingly, *ycaG*, *rgpE*, *ybcC*, and *ybcB* are all part of the same operon in *L. lactis* IL1403. This operon is probably involved in biosynthesis and transport of cell wall polysaccharides (WPS). Binding and infection studies showed that  $\phi$ 645 binds to and infects *L. lactis* Wg2, *L. lactis* IL1403, and *L. lactis* IL1403 strains with pGh9:ISS1 integration in *ycaG* and *rgpE*, whereas bIL170 binds to and infects only *L. lactis* IL1403 and cannot infect Wg2. These results indicate that  $\phi$ 645 binds to a WPS structure present in both *L. lactis* IL1403 and *L. lactis* Wg2, whereas bIL170 binds to another WPS structure not present in *L. lactis* Wg2. Binding of bIL170 and  $\phi$ 645 to different WPS structures was supported by alignment of the receptor-binding proteins of bIL170 and  $\phi$ 645 that showed no homology in the C-terminal part.

*Lactococcus lactis* is widely used in starter cultures for cheese production. Bacteriophage contamination during the fermentation process is a major problem, causing lysis of the starter bacteria and consequently slow or failed fermentation of the milk. Bacteriophage infection requires specific recognition between the phage receptor-binding protein (RBP) and the host cell receptor. A better understanding of this recognition mechanism should increase the possibility of preventing phage infection.

Bacterial receptors have been well studied in gram-negative bacteria, especially *Escherichia coli*. Phages attacking *E. coli* recognize either lipopolysaccharides or specific proteins of the outer membrane. For example, phage lambda initially interacts reversibly and then interacts irreversibly with the outer membrane protein LamB (32, 35), which facilitates the diffusion of maltose into the cell (10). A slightly more complex mechanism is utilized by phage T5, which initially binds reversibly to polymannose O antigens in lipopolysaccharides at the *E. coli* surface (13, 14) and then binds irreversibly to the ferrichrome transporter FhuA (5, 19). Finally, phage T4 binds reversibly with its long tail fibers to B-type lipopolysaccharides or to the outer membrane porin OmpC (15, 16, 28), whereupon the additional short tail fibers bind irreversibly to the lipopolysaccharide core region (27).

For gram-positive bacteria the information on phage receptors is sparser. However, phages attacking *L. lactis* seem to

bind initially to specific carbohydrate receptors exposed to the surface of the cell wall (29, 34, 40, 42). For many phages this binding step is reversible (29). Rhamnose, glucose, and galactose are often involved in this initial phage binding, as shown by the ability of these monosaccharides to competitively inhibit adsorption of several phages to cell surfaces (29, 40, 42). Binding to other carbohydrates, such as glucosamine and galactosamine, has been demonstrated for phage eb7 (20). For phages belonging to species c2, a secondary irreversible binding step requires binding to the membrane phage infection protein (PIP) (2, 12, 29, 41), while most phages of species 936, P335, and 949 do not use PIP as a secondary receptor (21); the only exception is 936-species phage kh (2, 29). Some of these phages may use other protein receptors in the membrane, as determined for 936-species phage sk1, which is able to bind to cell membranes deficient in PIP (2).

The aim of this work was to identify genes in *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* Wg2 that are important for adsorption of the 936-species phages bIL170 and  $\phi$ 645, respectively. Phage bIL170 and its host, *L. lactis* IL1403, are an excellent model system because the complete genome sequences are known (4, 7). In *L. lactis* IL1403 a biochemical or biological role has been assigned to 64.2% of the genes (4), and genes assumed to be involved in biosynthesis of surface polysaccharides and the PIP gene have been identified (2, 4). In bIL170 the RBP gene has recently been identified to be *orf20* (9). In contrast, the genome sequences of *L. lactis* Wg2 and phage  $\phi$ 645 are not available.  $\phi$ 645 was selected since it infects both *L. lactis* Wg2 and *L. lactis* IL1403, whereas bIL170 infects only *L. lactis* IL1403.

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TABLE 1. Phages, bacterial strains, and plasmid

Phage, bacterial strain, or plasmid	Relevant features	Reference or source
<b>Phages</b>		
bIL170 <sup>a</sup>	936-type lytic phage, propagated on IL1403 (AF009630)	7
bIL66	936-type lytic phage, propagated on IL1403	3
P008	936-type lytic phage, propagated on IL1403	24
P113G	936-type lytic phage, propagated on IL1403	23
P272	936-type lytic phage, propagated on IL1403	23
φ645	936-type lytic phage, propagated on IL1403 and Wg2	Chr. Hansen A/S
<b><i>E. coli</i> TG1</b>		
Transformation host		Stratagene, La Jolla, Calif.
<b><i>L. lactis</i> strains</b>		
IL1403 <sup>a</sup>	<i>L. lactis</i> subsp. <i>lactis</i> , plasmid free (AE005176)	4
Wg2	<i>L. lactis</i> subsp. <i>cremoris</i>	30
MG1614	St <sup>r</sup> Rif <sup>r</sup> derivative of <i>L. lactis</i> subsp. <i>cremoris</i> MG1363, plasmid free	11
<b>Plasmid</b>		
pGh9:ISS1	Used in insertional mutagenesis, Em <sup>r</sup>	25

<sup>a</sup> Phage or bacterial strain with known genome sequence. The GenBank accession number is indicated in parentheses.

Here we report that the genes identified, which are putatively involved in biosynthesis of cell wall polysaccharides in *L. lactis* IL1403 and *L. lactis* Wg2, are important for adsorption of phages bIL170 and φ645, respectively.

#### MATERIALS AND METHODS

**Bacteria, bacteriophages, and growth conditions.** The bacterial strains and bacteriophages used in this study are listed in Table 1.

*E. coli* TG1 was grown in Luria-Bertani medium (33) at 37°C with 200 μg of erythromycin per ml when appropriate. *L. lactis* strains were cultured at selected temperatures between 28 and 37.5°C in M17 broth (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) containing 0.5% glucose (*L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* MG1614) or 0.5% lactose (*L. lactis* subsp. *cremoris* Wg2). Erythromycin (2 or 5 μg/ml) was added when appropriate. Phages were propagated on their hosts cultured in media supplemented with 5 mM CaCl<sub>2</sub>.

**Electroporation procedure.** Electrotransformation of plasmid DNA into *E. coli* was performed essentially as described by Sambrook and Russell (33), while electroporation of *L. lactis* was performed as described by Holo and Nes (17).

**Insertional mutagenesis and selection for phage-resistant *L. lactis* strains.** Random insertional mutagenesis in *L. lactis* IL1403 and *L. lactis* Wg2 was carried out with the vector pGh9:ISS1 essentially as described by Maguin et al. (26). The vector system combined the insertion sequence ISS1 with a thermosensitive replicon, which allowed optimal replication of plasmids in the host at 28 to 30°C and integration of the entire plasmid between duplicated ISS1 sequences at temperatures at or above 37°C.

*L. lactis* strains containing the vector pGh9:ISS1 were grown overnight at 30°C with 5 μg of erythromycin per ml. After this, the cultures were diluted 100-fold in M17 broth with the appropriate carbohydrate but without erythromycin and incubated for 2.5 h at 30°C; this was followed by a temperature shift to 37°C for 2.5 h. Aliquots (200 μl) of the cultures were mixed with approximately 10<sup>9</sup> bIL170 or φ645 phages and imbedded in a soft agar layer as described for standard plaque assays. Erythromycin (2 μg/ml) was added to the agar media used for this assay. Colonies appearing after 2 days of incubation at 37°C were isolated and tested for phage sensitivity by cross-streaking on M17-Ca<sup>2+</sup> agar containing 0.5% glucose with the phage used for selection (18).

Excision of the pGh9:ISS1 vector from the chromosome of phage-resistant integrants, leaving a solitary ISS1 sequence in the chromosome, was carried out as described by Maguin et al. (26).

**Phage binding assay with SYBR Gold-stained phages.** Phages were SYBR Gold stained essentially as described by Noble and Fuhrman (30). A phage lysate having a titer of at least 10<sup>10</sup> PFU/ml was mixed with 20:1 (vol/vol) of a 1,000-fold-diluted SYBR Gold stock solution (Molecular Probes, Inc., Eugene, Oreg.) and incubated overnight in the dark at 4°C. Phages were mixed 1:1 with host cells grown to an optical density at 600 nm (OD<sub>600</sub>) of 0.6, and phage binding was examined by using a fluorescence microscope (Axioplan 2; Zeiss, Inc., Jena,

Germany) and a magnification of ×1,000 with blue light (450- to 490-nm) excitation. Phage binding to the host cells was indicated by a fluorescent halo around the cells.

**Phage adsorption assay.** Stationary-phase cultures of phage-resistant integrants of *L. lactis* IL-403 and *L. lactis* Wg2 grown at 37°C were diluted 100-fold in M17-Ca<sup>2+</sup> broth, grown to an OD<sub>600</sub> of 0.5, and infected with homologous phages at a multiplicity of infection of 0.001. The mixtures were incubated for 10 min at 30°C to allow binding of the phages to cell surfaces. After centrifugation (3 min, 15,000 × g, 4°C) the phage titer in the supernatant was determined by a standard plaque assay with the wild-type host strain. The percentage of adsorption was calculated as follows: [1 - (phage titer of supernatant after cells were removed/phage titer of a control reaction mixture without cells)] × 100.

**Southern analysis of integrants with reduced phage adsorption.** Southern analyses were carried out as described by Maguin et al. (26). Chromosomal DNA was purified with a DNeasy QIAGEN tissue kit (QIAGEN, Inc., Chatsworth, Calif.) as recommended by the manufacturer and was digested with EcoRI (New England Biolabs, Inc., Beverly, Mass.). Southern blotting was performed with a chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, England) by using pGh9:ISS1 as the probe.

**Rescue cloning and sequencing of pGh9:ISS1 flanking regions.** Chromosomal DNA from integrants digested with EcoRI (New England Biolabs) was treated with T4 DNA ligase (Roche Applied Science, Basel, Switzerland) as described by Maguin et al. (26) and was transformed into *E. coli* TG1 or *L. lactis* MG1614. Each transformation mixture was grown at 30°C with erythromycin selection. Rescued plasmids (pGh9:ISS1 including flanking chromosomal DNA) were purified from the transformants with a QIAprep spin plasmid miniprep kit (QIAGEN, Inc.) used as recommended by the manufacturer, except that *L. lactis* MG1614 initially was incubated with lysozyme (20 mg/ml) for 15 min at 37°C. Chromosomal DNA flanking pGh9:ISS1 was sequenced by using the reverse primer Gh9ISEcoR (Table 2), which annealed upstream in the ISS1 element.

**Purification of total RNA from integrants of *L. lactis*.** Integrants of *L. lactis* IL1403 were grown to an OD<sub>600</sub> of 0.6. Aliquots (1.5 ml) of the cultures were harvested by centrifugation at 20,000 × g for 5 min at 4°C and kept at -80°C. The harvested cells were mixed with 300 mg of glass beads (diameter, 106 μm; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) and 100 μl of lysis buffer supplemented with 0.7 μl of β-mercaptoethanol from an Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, Calif.). Cells were disrupted by shaking them for 40 s at 6.0 m/s with a FastPrep FP120 bead beater (Bio 101 Savant, Savant Instruments, Inc., Holbrook, N.Y.). After chilling on ice, the samples were centrifuged at 20,000 × g for 2 min at 4°C. Each supernatant was mixed with 500 μl of lysis buffer containing 3.5 μl of β-mercaptoethanol, and total RNA was isolated with the Absolutely RNA RT-PCR miniprep kit (Stratagene) used as recommended by the manufacturer.

**RT-PCR of total RNA.** Reverse transcription (RT)-PCR was carried out with total RNA by using the ProSTAR HF single-tube RT-PCR system (Stratagene) according to the manufacturer's instructions. As a control, RT-PCR was carried

TABLE 2. Primers used for PCR analyses in this study

Primer	Sequence (5'-3')	Position	Restriction site
Gh9ISEcoR	GAAGAAATGGAACGCTC	Near EcoRI site <sup>a</sup>	
ISS1F	TGTGATTATTGTCGCTGTTGG	Upstream in ISS1 <sup>a</sup>	
sk1orf16F	GCTCTAGAATAGAAAAAGAATCAGACAA <sup>b</sup>	13117 in sk1 <sup>c</sup>	XbaI
sk1orf20R	AACTGCAGAAAGTATGAGCGACAACCTCTC <sup>b</sup>	15469 in sk1	PstI

<sup>a</sup> In the vector pGh9:ISS1.

<sup>b</sup> The underlined sequences are restriction sites.

<sup>c</sup> The GenBank accession number for sk1 is AF011378.

out without reverse transcriptase to ensure that the purified RNA was free of contaminating DNA. The positions of primers used in RT-PCR to determine the size of the operon are shown in Table 3. To investigate transcription from the *ISS1* sequence, a forward primer (ISS1F [Table 2]) that annealed inside this sequence but downstream from the *ISS1* promoter was used.

**Monosaccharide inhibition of phage infection.** Rhamnose (0.5 M) or glucose (0.5 M) was added to the growth medium in infection experiments that included *L. lactis* IL1403 and phage bIL170 as described by Monteville et al. (29). The infection was monitored by spectrophotometric measurement at 600 nm.

**PCR amplification of the RBP gene in phage  $\phi$ 645.** The RBP gene in phage  $\phi$ 645 was PCR amplified by using primers sk1orf16F and sk1orf20R (Table 2) originally designed for PCR amplifying the RBP gene (*orf18*) in phage sk1 (9).

**DNA sequence analysis.** A DNA sequence analysis was performed with an ABI PRISM 310 genetic analyzer (Perkin-Elmer, Wellesley, Mass.). Sequences were assembled by using the ContigExpress program of the Vector NTI Suite 7 software package (Invitrogen, Carlsbad, Calif.). Sequences were aligned by using the ClustalX (version 1.81) program (37). BLAST searches (1) were conducted by using the National Center for Biotechnology Information homepage (<http://www.ncbi.nlm.nih.gov/BLAST/>). Transmembrane helices in proteins (22) were predicted by using the Center for Biological Sequence Analysis homepage (<http://www.cbs.dtu.dk/services/TMHMM/>).

**Nucleotide sequence accession number.** Sequence data for the RBP gene in phage  $\phi$ 645 have been deposited in the GenBank database under accession no. AY515015.

TABLE 3. RT-PCR analysis of the *L. lactis* IL-1403 region containing *ycaG*, *rgpE*, *ycbB*, and *ycbC*

Positions of primer pairs in IL-1403 <sup>a</sup>	GenBank accession no.	Genes	PCR product <sup>b</sup>	Size of PCR product (kbp) <sup>c</sup>
421 and 4838	AE006257	<i>ybjB</i> and <i>rmlC</i>	–	
1343 and 3008	AE006257	<i>rmlA</i> and <i>ybjF</i>	+	1.7
2822 and 4838	AE006257	<i>ybjF</i> and <i>rmlC</i>	+	2.0
3563 and 1260	AE006257/58	<i>rmlB</i> and <i>rgpA</i>	+	2.9
212 and 2657	AE006258	<i>rgpA</i> and <i>rgpC</i>	+	2.5
2314 and 5575	AE006258	<i>rgpC</i> and <i>ycaG</i>	+	3.2
2314 and 5753	AE006258	<i>rgpC</i> and <i>rgpE</i>	+	3.4
6481 and 7855	AE006258	<i>rgpE</i> and <i>rgpF</i>	+	1.3
6637 and 9866 <sup>d</sup>	AE006258	<i>rgpF</i> and <i>ycbA</i>	+	3.2
8476 and 1875	AE006258/59	<i>ycbA</i> and <i>ycbD</i>	+	3.8
883 and 5344	AE006259	<i>ycbC</i> and <i>ycbG</i>	+	4.4
4973 and 9013	AE006259	<i>ycbG</i> and <i>tagD1</i>	+	4.0
7811 and 9318	AE006259	<i>ycbJ</i> and <i>yccB</i>	+	1.5
7811 and 992	AE006259/60	<i>ycbJ</i> and <i>guaB</i>	–	

<sup>a</sup> The numbers are the positions of the first nucleotides in the 5' ends of the primers used with DNA sequences of *L. lactis* IL-1403. The number system is the system for the previously published sections of the *L. lactis* IL-1403 genome. The primers were 20 bp long.

<sup>b</sup> –, no PCR product; +, PCR product evident.

<sup>c</sup> The results were obtained by performing overlapping RT-PCR with total RNA from the integrants of *L. lactis* IL-1403.

<sup>d</sup> The combination was tested with wild-type IL-1403 total RNA, and negative results were obtained.

## RESULTS

**Isolation of integrants of *L. lactis* IL1403 and *L. lactis* Wg2 with reduced phage adsorption.** To identify genes involved in phage binding in *L. lactis* strains IL1403 and Wg2, a panel of mutants carrying random chromosomal integrations of plasmid pGh9:ISS1 was generated. Subsequently, phage-resistant mutants of *L. lactis* IL1403 and *L. lactis* Wg2 were selected on the basis of the ability to grow in the presence of the bIL170 and  $\phi$ 645 phages, respectively. This selection resulted in several hundred phage-resistant mutants of *L. lactis* Wg2 and 10 phage-resistant mutants of *L. lactis* IL1403. Phage-resistant integrants of *L. lactis* Wg2 appeared at a rate of  $3 \times 10^{-6}$ , whereas control experiments showed that spontaneous phage-resistant mutants were obtained at a rate of  $2 \times 10^{-7}$ . In *L. lactis* IL1403 phage-resistant integrants were obtained at a rate of  $5 \times 10^{-6}$ , whereas no spontaneous mutants were obtained at a frequency of  $>10^{-7}$ .

To screen for phage-resistant integrants with a reduced ability to adsorb phages, adsorption assays were carried out. Fifteen randomly selected phage-resistant integrants of *L. lactis* Wg2 were examined, and 10 adsorption-deficient integrants were retained due to low adsorption (0 to 23%) of added  $\phi$ 645 phage (Table 4). All 10 phage-resistant integrants of *L. lactis* IL1403 showed a reduced ability to adsorb bIL170 phage (0 to 27% adsorption) (Table 4). In contrast, the levels of adsorption of bIL170 and  $\phi$ 645 to their indicator strains, *L. lactis* IL1403 and *L. lactis* Wg2, respectively, were approximately 97%.

The reduced abilities of the phage-resistant integrants of *L. lactis* IL1403 and *L. lactis* Wg2 to adsorb phages bIL170 and  $\phi$ 645, respectively, were confirmed by a binding assay in which SYBR Gold-stained phages were used. The cells were subsequently observed by fluorescence microscopy to detect phage binding. None of the integrants showed visible phage binding, whereas binding to the wild-type strains was documented by the appearance of bright fluorescent halos.

**Southern blot analysis of pGh9:ISS1 integrants with reduced phage adsorption.** The 10 adsorption-deficient integrants of *L. lactis* IL1403 were examined by Southern blot analysis by using pGh9:ISS1 as the probe to identify different integrant types. Four different integrant types of *L. lactis* IL1403 were identified on the basis of the band patterns appearing in the Southern blot (Fig. 1). Likewise, two different integrant types of *L. lactis* Wg2 were identified (data not shown). The appearance of several integrants with similar band patterns in the Southern blots could have indicated that they were daughter cells of the same original integrant.

TABLE 4. Phage adsorption assay for bIL170 and  $\phi$ 645 with pGh9:ISS1 integrants of *L. lactis* IL1403 and *L. lactis* Wg2, respectively

Strain	Adsorption of bIL170 (%)	Adsorption of $\phi$ 645 (%)
<i>L. lactis</i> IL1403	97	
Integrand 1	20	
Integrand 2	20	
Integrand 3	10	
Integrand 4	27	
Integrand 5 <sup>a</sup>	20	
Integrand 6	13	
Integrand 7 <sup>a</sup>	10	
Integrand 8 <sup>a</sup>	0	
Integrand 9 <sup>a</sup>	26	
Integrand 10	9	
<i>L. lactis</i> Wg2		97
Integrand 1 <sup>a</sup>		0
Integrand 2		22
Integrand 3		23
Integrand 4		0
Integrand 5		5
Integrand 6		19
Integrand 7		0
Integrand 8 <sup>a</sup>		17
Integrand 9		0
Integrand 10		10

<sup>a</sup> Integrand with identified integration site of pGh9:ISS1 (Fig. 2).

**Analysis of pGh9:ISS1 insertion sites.** The chromosomal integration sites of pGh9:ISS1 were determined in the four integrant types of *L. lactis* IL1403 and in the two integrant types of *L. lactis* Wg2 by rescue cloning of pGh9:ISS1 and flanking chromosomal DNA. Sequence analysis showed that insertion in *L. lactis* IL1403 had occurred at three different sites in the *rgpE* gene and at one site in an open reading frame upstream from *rgpE*, designated *ycaG* (Fig. 2). *rgpE* encodes a putative glycosyltransferase (4), whereas the function of *ycaG* is unknown.

For integrants of *L. lactis* Wg2 the derived amino acid sequence of the rescued flanking sequence from integrant 1

showed 77% identity to YcbB of *L. lactis* IL1403 (Fig. 2), a putative glycosyltransferase. The amino acid sequence derived from the flanking region of integrant 8 showed 62% identity to YcbC of *L. lactis* IL1403 (Fig. 2), a protein with no known function.

**Transcription analysis.** In the genome of *L. lactis* IL1403 the *rgpE*, *ycaG*, *ycaB*, and *ycaC* genes are located within a 6.4-kbp region (4), indicating that they could be part of an operon. Therefore, integrants of *L. lactis* IL1403 were examined for polar effects on downstream transcribed genes by RT-PCR. When pGh9:ISS1 was inserted into the chromosome, no polar effects on downstream transcription were observed (Fig. 3A).

To examine the effects of a solitary ISS1 element integrated into the chromosome on the downstream transcription, pGh9:ISS1 was excised from the chromosome. When a primer pair annealing to chromosomal sequences flanking the solitary ISS1 sequence was used in RT-PCR, no PCR product was produced (Fig. 3B). In contrast, when the RT-PCR analysis was carried out with a primer pair annealing inside the ISS1 sequence and downstream of the ISS1 insertion, a PCR product was generated (Fig. 3B). Hence, transcription was knocked out by integration of the ISS1 sequence but was restarted from inside the ISS1 sequence, indicating that a promoter reading into the region downstream of the insertion was present in the ISS1 sequence.

As control, RT-PCR was carried out without reverse transcriptase in the reaction mixture. The results showed that PCR products were not obtained without reverse transcriptase in the reaction mixture (Fig. 3A).

To determine if *rgpE*, *ycaG*, *ycaB*, and *ycaC* in fact were part of an operon, overlapping RT-PCR was carried out with total RNA isolated from the integrants of *L. lactis* IL1403 (Table 3). Interestingly, *rgpE*, *ycaG*, *ycaB*, and *ycaC* were found to be part of the same operon. The operon was determined to be approximately 25 kbp long and contained 24 genes, starting with *rmlA* and ending with *tagD1* (Fig. 4). Attempts to conduct the same experiments with wild-type IL1403 RNA were unsuccessful as RNA containing the complete operon could not be purified. The demarcation of the operon was confirmed by identification of a hairpin-loop structure followed by a promoter-like sequence in the intergenic region upstream from *rmlA*. At the distant part of the operon an additional hairpin-loop structure was located in the intergenic region between *tagD1* and the subsequent gene, *ycaB*. In spite of the hairpin-loop structure, RT-PCR analysis showed that the transcript continued into *ycaB* but that the downstream gene, *guaB*, was not part of the operon (Table 3). However, due to the opposite orientation of *ycaB* we do not expect that this part of the transcript is translated into a protein. As the size of the operon was obtained by RT-PCR, the possibility that there are additional transcription start sites within the operon cannot be excluded.

**Monosaccharide inhibition of phage infection.** To clarify if glucose or rhamnose was part of the receptor for bIL170 in *L. lactis* IL1403, infection experiments were conducted with 0.5 M glucose or 0.5 M rhamnose in the growth medium. Rhamnose delayed the lysis of the culture for approximately 1 h, whereas glucose did not inhibit the lysis.

**Infection and binding abilities of phages bIL170 and  $\phi$ 645.** The abilities of bIL170 and  $\phi$ 645 to infect and bind to *L. lactis*

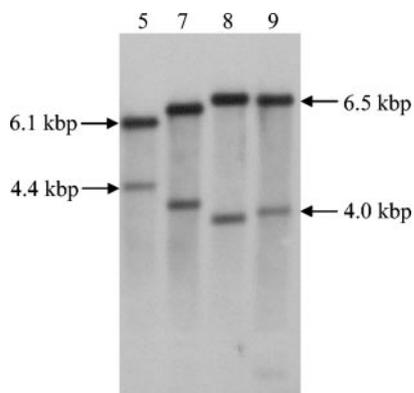


FIG. 1. Southern analyses of chromosomal DNA from selected integrants of *L. lactis* IL1403 with reduced binding of phage bIL170. Chromosomal DNA was digested with EcoRI, and pGh9:ISS1 was used as a probe. The two bands in each lane represent chromosomal DNA with a flanking pGh9:ISS1 sequence and a flanking ISS1 sequence (25). The numbers at the top indicate the integrants used (see Table 4).

**A**  
*ycaG*  
MHLPGAIGVLIARLIYPSLGIMDYGGRIANLICFSLIFYFLIKKNEHAKWSMLIFMVGGIQQIFSPSYDVVSFLVFSAFVNVLSDL  
VRIEKIRDVGLKKAYTIFLICSFYFIKSNYIFAFFALLGLPMLYRPVIDKVRKLSLGGKTFLSMLIIGHSVAYLFLNKKMSIFTIKK  
FIENYMNVELMGNNAKQLWQVVPPTLPFVNLFILILFVMMGELKATWATGTVIIIFSLTYLVNWFVGFAGFFIDSASLASTNL  
∇5  
QGRYLSPLFFFFVQNLGKKNFTMSEKSVRRLSVWTVIIISVLVYLVVTFYRSYVLKITPTWTNNA

*rgpE*  
MLDKISVVVTCHNHEAYIEQCLRSVFAQTYRNIELLVFNDGSTDQSGQVIEKILKESPFSEYFSEENRGIVSIRNDALTKIK  
∇7  
∇8 ∇9  
GDFLLFVDSNYLDDQYIEILHQLQEQNVDIAYCQLWDFVNSRQVLRDDLEFSLDKELEGNLIDMSSLVVRVSKILNVR  
FDEQLNHKALEDYDFWLSLILINHAKPIFVKGIKLNRYVLTNSTRTERGNWDKYYSFYLLLEKHQTLPLKEVLKASQANVKIWL  
KSYLESQQLTENILKIKEKDKHISNLQREIDLLKSLAAKEDKTKAELEEKQEIIDIKNSKYFLQKLGKN

**B**  
*ycbB*  
MNKQRILLIIPAYNESEGIKVVVEVVKQYISKIELEIDYVINDGSTDNEEEVLREYNITHIELIQNLGIGGAVQTGYMYALENSY  
DIAVQFDGQDGHDIHSLPNLEPILNDEADFTVGSRFLDENSEFKSSSRQLGKIKLSSLIYMSSKIKIKDVTSGYRAGNRKAIEQ  
∇1  
FVKRYPRQYPEPESYMHLFSKNIRVKEVGVRMFERTTGVSSINLIKGVNYMVSLSLAILASSILGKEK

*ycbC*  
∇8  
MIMPIQLRLLAIIAVALVFFVYMVRLVRKDKAEIRHMLKWLVLALILIGAIFFEMGSRIAHFFGIQTLTSLALFILVGIILLISLK  
YQMSLISAEKQIKNLIQEVSLANKKIKDLERKNDNKE

FIG. 2. Integration sites of pGh9:ISS1 in integrants of *L. lactis* IL1403 (A) and *L. lactis* Wg2 (B) resistant to phages bIL170 and φ645, respectively. The integration sites in the resulting proteins are indicated by open triangles and numbers that correspond to the integrants shown in Table 4 and in Fig. 1. For *L. lactis* Wg2 the approximate integration sites are indicated in the homologous *L. lactis* IL1403 proteins.

IL1403, *L. lactis* Wg2, and integrants with reduced phage adsorption were determined by plaque assays and binding assays performed with SYBR Gold-stained phages, respectively, to clarify if the two phages use different binding sites. φ645 was able to bind to and infect both *L. lactis* IL1403 and the pGh9:ISS1-integrants of *L. lactis* IL1403 with reduced adsorption of bIL170 (Table 5). In contrast, bIL170 did not bind to and infect *L. lactis* Wg2 or the integrants of this strain with reduced

adsorption of φ645. Hence, the binding site for φ645 is present in both *L. lactis* IL1403 and *L. lactis* Wg2 and is different from the binding site used by bIL170, which is present only in *L. lactis* IL1403. A puzzling observation was that the plaques of φ645 infecting IL1403 integrants were more than twice as large as those of φ645 infecting wild-type strain *L. lactis* IL1403 (Table 5).

**Infection and binding abilities of phages bIL66, P008, p272, and P113G.** Phages bIL66, P008, p272, and P113G have an RBP and a host range similar to those of bIL170 (9). The infection and binding abilities of the phages with *L. lactis* IL1403 and integrants of *L. lactis* IL1403 with reduced adsorption of bIL170 were determined by plaque assays and binding assays performed with SYBR Gold-stained phages, respectively. The results showed that all the phages infected *L. lactis* IL1403, whereas they neither infected nor bound to integrants of *L. lactis* IL1403.

**Alignment of the RBP in bIL170 and φ645.** The RBP genes have recently been identified in bIL170 and some other phages of species 936 (9). The primers used for PCR amplification of the RBP genes in these phages were used with phage φ645. The PCR product was sequenced, and the derived amino acid sequence was compared to the amino acid sequence of the RBP from bIL170 to determine if φ645 has an RBP that is different from that of bIL170. In Fig. 5 the deduced amino acid sequences of the RBPs in φ645 and bIL170 are aligned. In the approximately 150-amino-acid N-terminal part of the RBPs 80% identity was found between φ645 and bIL170, whereas in the approximately 100-amino-acid C-terminal part of the RBPs no homology was found. Hence, the RBPs from φ645 and bIL170 had an N-terminal homologous part and a C-terminal nonhomologous part.

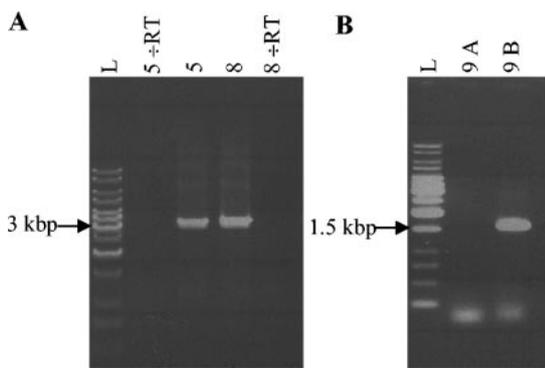


FIG. 3. RT-PCR of phage-resistant integrants of *L. lactis* IL1403. (A) Integrants 5 and 8 (see Table 4 and Fig. 2) with pGh9:ISS1 integrated into the *ycaG* and *rgpE* genes, respectively. Primers annealed downstream from the integrated pGh9:ISS1 in *rgpF* and *ycaA* (nucleotides 6637 and 9866; accession no. AE006258) (Table 3). (B) Integrant 9 (see Table 4 and Fig. 2) with a solitary ISS1 sequence integrated into the *rgpE* gene. Lane 9A, primers annealing on each side of the ISS1 sequence in the *rgpC* and *rgpF* genes (nucleotides 2314 and 7835; accession no. AE006258) (Table 3); lane 9B, primers annealing in the ISS1 sequence (ISS1F [Table 2]) and in the *rgpF* gene (nucleotide 7855; accession no. AE006258) (Table 3); lane L, 1-kb DNA ladder. RT, reverse transcriptase.

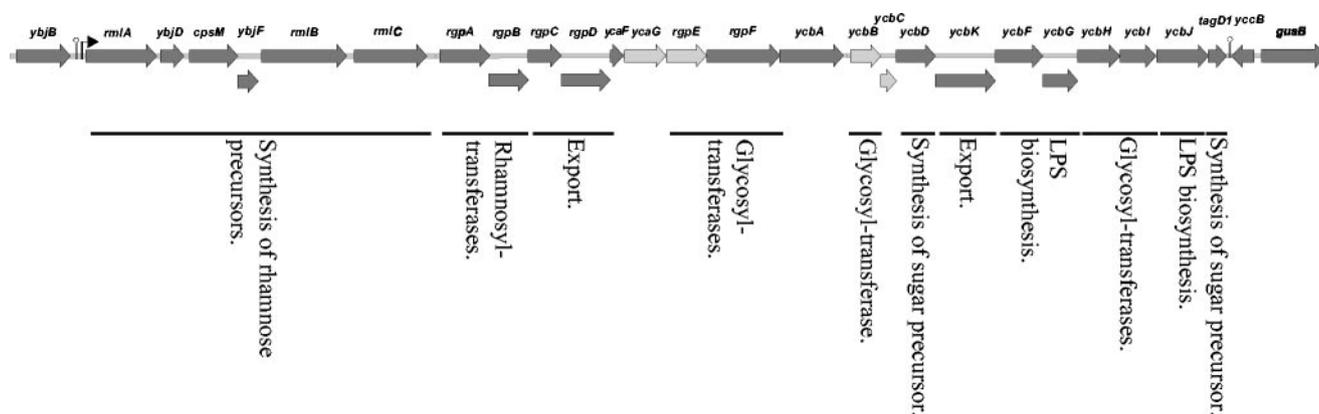


FIG. 4. Operon in *L. lactis* IL1403 containing the *ycaG*, *rgpE*, *ycbB*, and *ycbC* genes. The light grey arrows represent genes interrupted in integrants of *L. lactis* IL1403 (*ycaG* and *rgpE*) and *L. lactis* Wg2 (*ycbB* and *ycbC*) resistant to phages bIL170 and  $\phi$ 645, respectively. The displaced arrows indicate overlapping open reading frames. The bent arrow indicates the promoter, and the hairpin symbols indicate the terminators. LPS, lipopolysaccharide.

## DISCUSSION

In this study we showed that the *ycaG* and *rgpE* genes in *L. lactis* IL1403 are important for adsorption of phage bIL170 to the cell surface. Likewise, we identified two genes in *L. lactis* Wg2 that are important for adsorption of phage  $\phi$ 645, which show high levels of similarity to *ycbB* and *ycbC* of *L. lactis* IL1403. Since  $\phi$ 645 infects *L. lactis* IL1403, we believe that it is likely that *ycbB* and *ycbC* are important for adsorption of  $\phi$ 645 in this strain.

Transcriptional analyses of the integrants revealed no polar effects on downstream transcribed genes due to insertion of pGh9:ISS1 into the chromosome (Fig. 3A), confirming that *ycaG*, *rgpE*, *ycbB*, and *ycbC* are involved in phage binding. In all six integrants investigated, the ISS1 sequences were orientated in the same direction as the interrupted genes, suggesting that the opposite direction results in polar effects that are lethal to the cells. pGh9:ISS1 therefore turned out to be an excellent mutagenesis tool that did not have polar effects on downstream transcribed genes. Transcription analyses of an integrant with a solitary ISS1 sequence in the chromosome (Fig. 3B) indicated that transcription restarts from a promoter inside the ISS1 sequence. This is consistent with a functional transcription promoter in the ISS1 sequence, which is localized just upstream from an open reading frame that probably encodes a transposase (6).

The two interrupted genes in *L. lactis* IL1403 (*ycaG* and *rgpE*), as well as *ycbB* and *ycbC*, which have high levels of similarity to the genes interrupted in phage-resistant integrants of *L. lactis* Wg2, turned out to be part of the same operon (Fig.

4). The operon contains genes assumed to be involved in biosynthesis and export of cell wall polysaccharides (WPSs) (4). This is in accordance with adsorption of lactococcal phages to specific carbohydrates in the cell wall (20, 29, 40, 42). Previously, genes involved in biosynthesis and export of WPSs have been identified in *Streptococcus mutans* (38, 39, 44), but from the investigations it is not clear if all the genes are located close to each other on the *S. mutans* genome or even are part of the same operon.

The function of YcaG is unknown, but predictions of transmembrane helices (see Material and Methods) in the protein suggest that it could be a membrane protein with seven transmembrane helices. We speculate that YcaG assists in transport of specific WPSs or parts of WPSs.

RgpE showed homology to several glycosyltransferases, but the specificity of the enzyme is not clear. The N-terminal two-thirds of RgpE have 24% identity and 46% similarity with RgpE of *S. mutans*. The *S. mutans* gene encodes a glycosyltransferase involved in glucose side chain formation in alternating  $\alpha$ -1,2- and  $\alpha$ -1,3-linked L-rhamnosyl polymers in WPSs (44). We do not believe that it is likely that RgpE in *L. lactis* IL1403 is involved in glucose side chain formation, as 0.5 M glucose did not competitively inhibit infection of *L. lactis* IL1403 by bIL170. Furthermore, no homology to RgpE from *S. mutans* was found in the C-terminal part of the protein. In contrast, 0.5 M rhamnose delayed the infection slightly. This is consistent with the putative functions of several upstream genes in the operon (Fig. 4) involved in synthesis of rhamnose precursors and rhamnosyl transferases (4) and with the occur-

TABLE 5. Infection and binding abilities of phages bIL170 and  $\phi$ 645<sup>a</sup>

Phage	<i>L. lactis</i> IL1403	<i>L. lactis</i> IL1403 integrant in <i>rgpE</i> <sup>b</sup>	<i>L. lactis</i> IL1403 integrant in <i>ycaG</i> <sup>b</sup>	<i>L. lactis</i> Wg2	<i>L. lactis</i> Wg2 integrant in <i>ycbB</i> <sup>b</sup>	<i>L. lactis</i> Wg2 integrant in <i>ycbC</i> <sup>b</sup>
bIL170	+ (2) <sup>c</sup>	–	–	–	–	–
645	+ (2)	+ (5)	+ (5)	+ (1)	–	–

<sup>a</sup> Infection was determined by plaque assays, and binding ability was determined by using SYBR Gold-stained phages. +, positive; –, negative.

<sup>b</sup> Integrants of *L. lactis* IL-1403 and *L. lactis* Wg2 with reduced adsorption of bIL170 and  $\phi$ 645, respectively.

<sup>c</sup> The numbers in parentheses are the diameters (in millimeters) of the plaques appearing in the plaque assays.



FIG. 5. Alignment of amino acid sequences of the RBPs in ϕ645 and bIL170. The stars above the sequences indicate positions which have a single, fully conserved residue. The alignment scores are shown below the ruler.

rence of rhamnose in the cell wall of an *L. lactis* strain (40). It appears likely that the WPSs in *L. lactis* IL1403 have a rhamnosyl backbone, as described previously for *S. mutans* (44), and that bIL170 interacts partially with this backbone. However, carbohydrate side chains added to the putative rhamnosyl backbone by RgpE activity are probably crucial for phage binding.

YcbB in *L. lactis* IL1403 showed homology to several glycosyltransferases, which were different from the glycosyltransferases with homology to RgpE. In addition, National Center for Biotechnology Information pairwise BLAST analysis (36) of RgpE and YcbB showed no homology, suggesting that the glycosyltransferase encoded by *ycbB* adds different carbohydrate side chains to the putative rhamnosyl backbone than the glycosyltransferase encoded by *rgpE* adds. Based on these indications, at least two different types of WPS structures are present in the cell wall of *L. lactis* IL1403. Coexistence of different WPS structures in the same strain has previously been described by Delcour et al. (8).

Finally, the function of the small protein YcbC is also unknown. Prediction of transmembrane helices (see Materials and Methods) suggested that YcbC could be a membrane protein with three transmembrane helices. As suggested for YcaG, YcbC may assist in biosynthesis or transport of specific WPSs or parts of WPSs.

It was observed that all of the phage-resistant integrants grew slightly slower than the wild-type strains and that some of the cells were elongated and deformed. In addition, cultures of *L. lactis* Wg2 integrants formed aggregates at the bottoms of the tubes during growth, which was not observed for the wild type. These observations indicate that WPS affects the structure of the cell wall and the cell surface characteristics. It was observed that the plaques after infection of integrants of *L. lactis* IL1403 with ϕ645 were more than twice as big as the plaques when the wild type was infected (Table 5). We speculated that the cell wall in integrants of *L. lactis* IL1403 is more fragile, which facilitates lysis and liberation of phages by the ϕ645-produced lysin. Larger plaques were also seen when glycine was incorporated into the growth media (23). Glycine has previously been shown to weaken the bacterial cell wall (17). The functionality of WPS is far from understood, but Yamashita et al. (43) found that knockout of *rmlB* in *S. mutans*, which prevents synthesis of WPSs, resulted in increased sensitivity to acidic pH, an elevated temperature, and high osmolarity, suggesting that WPSs stabilize the cell wall under extreme environmental conditions.

As ϕ645 and bIL170 seem to recognize and adsorb to dif-

ferent structures in the cell wall, it was expected that they differ in their RBPs. The phages showed homology in the N-terminal part of the RBPs but no homology in the C-terminal part. The homologous N-terminal part of the RBPs in bIL170 and ϕ645 was found to be homologous to the N-terminal part of RBPs from the following other phages of species 936: ϕ7 (GenBank accession number AF539450), p272 (AF539443), P113G (AF539451), sk1 (*orf18*) (AF011378), jj50 (AF539448), p2 (AF539441), jw30 (AF539452), jw31 (AY145884), jw32 (AY145885), P008 (AF539449), fd13 (AF539444), ϕ936 (AF539446), ϕ712 (AF539442), and P475 (AF539447). This large group of phages has different host ranges despite the homologous N-terminal part of their RBPs (Dupont, unpublished results). We therefore speculated that the N-terminal part of RBPs could be involved in binding to a conserved protein at the distal part of the phage tail and probably is not essential for host recognition. It has previously been shown that lactococcal phages that differ in the C-terminal part of the RBPs bind to different host strains (9). In that study two types of RBPs were identified: the sk1-type of RBPs, which were found in phages p2, fd13, jj50, ϕ7, and sk1, and the bIL170 type of RBPs, which were found in phages P008, P113G, p272, bIL66, and bIL170. As determined by BLAST searches (1), the nonhomologous C-terminal part of the ϕ645 RBP showed 72% identity to the RBP from phage ϕ712, indicating that there is a third type of RBPs in 936-species phages. Interestingly, it was found that like bIL170, the phages bIL66, P008, p272, and P113G infect *L. lactis* IL1403, whereas they neither bind to nor infect the integrants of *L. lactis* IL1403 exhibiting reduced adsorption of bIL170. These results confirm that *ycaG* and *rgpE* in *L. lactis* IL1403 are important for adsorption of phages with an RBP similar to the RBP of bIL170.

In conclusion, this study indicated that phages bIL170 and ϕ645 recognize and bind to different WPS structures in the cell wall. Loss of biosynthesis or export of WPS structures or parts of these structures is apparently sufficient for total inhibition of phage infection. We speculated about why integration of pGh9:ISS1 was not obtained in genes encoding membrane proteins with a possible function as a secondary receptor, like PIP (29). The method used cannot explain this observation, since PIP mutants previously have been obtained for *L. lactis* MG1614 by the same method (Stuer-Lauridsen, unpublished results). For *L. lactis* IL1403 and *L. lactis* Wg2 it is therefore possible that knockout of the genes encoding the secondary membrane receptors for bIL170 and ϕ645 is lethal for the cell or that a secondary membrane receptor does not exist. The latter proposal is supported by investigations with phage P008,

which binds irreversibly to a carbohydrate component embedded in the peptidoglycan matrix of *L. lactis* F7/2, resulting in DNA release (34).

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