Identification, Characterization, and Expression of a Second, Bicistronic, Operon Involved in the Production of Lactocin S in *Lactobacillus sakei* L45

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Through the analysis of spontaneous insertion mutants of *Lactobacillus sakei* L45, a second operon involved in lactocin S production was identified and characterized. The new, bicistronic unit, termed *lasXY*, is situated immediately upstream of the previously characterized nine-open reading frame (ORF) lactocin S operon (*lasA-W*) and is transcribed in the opposite direction. The proximal of the two newly identified genes, *lasX*, specifies a 285-residue protein that is similar to a group of proteins with reported gene regulation functions in gram-positive bacteria. It was demonstrated that the *lasX* mutants have a strongly reduced level of *lasA* and *lasA-W* mRNA, thus indicating the likely cause of the Bac^- phenotype of these mutants. The second ORF in the operon, *lasY*, specifies a 300-residue ABC transporter homolog, the function of which is currently obscure. Transcription initiation mapping of the *lasXY* operon demonstrates that the two lactocin S promoters overlap such that both transcripts initiate within the -35 region of the oppositely oriented promoter. This organization of promoters is unique among this group of regulons and may constitute a modulatory site in the proposed *LasX*-dependent expression of *lasA* and downstream genes.

Lactocin S (22, 23), which is produced by some *Lactobacillus sakei* strains, belongs to the group of antimicrobial peptides known as lantibiotics (35). Common to all lantibiotics (for a recent review on biosynthesis and biological activities, see reference 31) is the presence of one or more of the ring-forming residues lanthionine and/or methyl-lanthionine, which are generated through a sequence of posttranslational modifications of precursor peptides. The lantibiotics are divided into subcategories, type A and B, based on molecular shape and biological activities. Type A peptides, which constitute the majority of known lantibiotics (including lactocin S), are elongated, flexible molecules and probably have in common a membrane-disrupting (pore-forming) mode of action. Structural heterogeneity is, however, observed within both subclasses, comprising differences in the number of thioether rings and their location relative to each other, as well as the presence of additional amino acid modifications. Biosynthetic functions, such as prepeptide processing, may also vary between different lantibiotic systems, as may functions such as producer self-protection (immunity) and genetic regulation of production.

The structural and biosynthetic heterogeneity among the lantibiotics is reflected in the complexity of the genetic loci, which may contain as many as 11 (e.g., nisin producers [6]) or as few as 6 (e.g., lactocin 481 producers [29, 30]) genes dedicated to the production of and protection against a particular lantibiotic. The specific genes may be organized in one or more transcriptional units, but they form a single, compact gene cluster in all known cases.

We have previously reported the sequence and expression of a nine-open reading frame (9 ORF) lactocin S (*lasA-W*)

operon, containing the pre-lactocin S encoding gene, *lasA*, and candidate genes for functions such as peptide export (*lasT*), dehydration-thioether bridge formation (*lasM*), and prepeptide processing (*lasP*) (38). For two of these genes, *lasM* and *lasT*, the tentative functional assignments are supported by the Bac^- phenotype of spontaneous mutations caused by the insertion of the insertion (IS) element *IS1163* (39). The translation of *lasJ*, which is the penultimate ORF in the operon, is similar to the lantibiotic immunity factors PepI and EciI (11), suggesting that *lasJ* may be involved in producer self-protection in the lactocin S system. The four remaining ORFs in the *lasA-W* operon are either unique (i.e., no significantly similar sequences were found in publicly available nucleic acid or protein databases) or they encode proteins that are novel in a lantibiotic context. We have, so far, not identified mutations in any of the latter genes, and thus their possible significance in determining the lactocin S phenotype, if any, remains to be established.

Recently, a second transpositionally active IS element, *IS1520*, was identified in the lactocin S-producing strain (40). As is the case with the related *IS1163* element, *IS1520* causes the cessation of bacteriocin production by transposition to the pCIM1 *las* locus. In one of the isolated mutants, the element interrupted the third ORF (*lasN*, previously known as *orf239*) in the *lasA-W* operon, thereby suggesting a role for this gene in maintaining the lactocin S phenotype. The remaining *IS1520* insertions, however, mapped outside the characterized *las* operon, indicating the presence of an additional gene(s) involved in lactocin S production. Below, we present an analysis of these mutants, demonstrating that the affected gene, *lasX*, is necessary for normal expression of the *lasA-W* operon. *lasX* is the proximal gene in the bicistronic operon *lasXY*, which is located upstream of *lasA-W* and transcribed in the opposite direction. The absence of relevant genetic information in the

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TABLE 1. Bacterial strains, bacteriophages, and plasmids used in this study

Strain, plasmid, or phage	Genotype and/or description	Comment and/or other features ^a	Source and/or reference
Strains			
<i>E. coli</i>			
DH5 α	F ⁻ ϕ 80d <i>lacZ</i> ΔM15Δ(<i>lacZYA-argF</i>)U169 <i>endA1 recA1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>deoR thi-1 supE44 λ-gyrA96 relA1</i>	Cloning host (blue-white selection)	Gibco-BRL
LE392	F ⁻ e14 ⁻ (McrA ⁻) <i>hsdR514</i> (r _k ⁻ m _k ⁺) <i>supE44 supF58 lacY1</i> or Δ(<i>lacIZY</i>)6 <i>galK2 galT22 metB1 trpR55</i>	Propagation of bacteriophage λ and derivatives	21
P2392	LE392(P2)	As for LE392; P2 lysogeny allows <i>spi</i> selection of λ recombinants	37
<i>L. sakei</i>			
L45-2.1	L45 derivative cured of pCIM2	Bac ⁺ Imm ⁺ (lactocin S wild type)	22
L45-3.1	L45 derivative cured of pCIM1	Bac ⁻ Imm ⁻	22
L45-5.1	<i>lasM::IS1163</i>	Bac ⁻ Imm ⁺	38
L45-5.2	<i>lasT::IS1163</i>	Bac ⁻ Imm ⁺	38
L45-5.3	<i>lasX1::IS1520</i>	Bac ⁻ Imm ⁺	This study
L45-5.6	<i>lasX2::IS1163</i>	Bac ⁻ Imm ⁺	This study
L45-5.7	<i>lasX3::IS1520</i>	Bac ⁻ Imm ⁺	This study
L45-5.8	<i>lasN::IS1520</i>	Bac ⁻ Imm ⁺	40; this study
Plasmids			
pUC18	High-copy-number <i>E. coli</i> cloning vectors	None	48
pGEM7-Zf(+)		MCS flanked by T7 and SP6 promoters	Promega
pCIM1	lactocin S-encoding plasmid		22
plas7.5-1	pUC derivative carrying a 7.45-kb pCIM1 <i>Hind</i> III fragment	Sequencing template; nt 5445 to 12887	38
plas3.5-1	pGEM-derivative carrying a 3.5-kb <i>Eco</i> RI- <i>Hind</i> III fragment subcloned from λ-las43	Sequencing template; nt 1 to 3576	This study
λ derivatives			
λ-DASHII	Replacement vector	MCS flanked by T7 and T3 promoters	Stratagene
λ-las43	λ-DASHII derivative ^b	Source of plas3.5-1 insert	38; this study
λ-las44	λ-DASHII derivative ^b	PCR-sequencing template; nt 13431 to 15175	38; this study
λ-las46	λ-DASHII derivative ^b	PCR-sequencing template; nt 5048 to 5495	38; this study
λ-las62	λ-DASHII derivative ^b	PCR-sequencing template; nt 4173 to 5140	38; this study
λ-las63	λ-DASHII derivative ^b	PCR-sequencing template; nt 3518 to 4220 and nt 14992-15996	38; this study
λ-las103	λ-DASHII derivative ^b	PCR-sequencing template; nt 12694 to 13707	38; this study

^a nt, nucleotides.^b That is, a DASHII derivative carrying overlapping sections of the pCIM1 *las* region.

regions flanking the two operons, suggests that *lasXY* and *lasA-W* constitute a complete lactocin S locus.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains, plasmids, and phage lambda derivatives used here are listed in Table 1. MRS liquid or solid (1.5% agar) media were used for propagation of the *Pediococcus acidilactici* PAC1.0 (lactocin S sensitive indicator strain) and *L. sakei* strains at 30°C. Bacteriocin assays (deferred plate assay or microtiter plate assay) were performed as described previously (22). *Escherichia coli* strains were cultivated in Luria-Bertani medium (supplied with 100 μg of ampicillin/ml when appropriate for selection) at 37°C or as specified by the supplier of the lambda DASHII cloning vector (Stratagene Cloning Systems, La Jolla, Calif.). Plasmid DNA was purified in CsCl gradients subsequent to a standard alkaline lysis protocol (32) by using elevated lysozyme concentrations (10 mg/ml) when plasmid was isolated from *L. sakei*. Phage lambda DNA was isolated as suggested by the vector supplier.

DNA manipulations, amplification, and sequencing. Enzymes used for restriction digestion, cloning, amplification, and sequencing of DNA were purchased from Promega (Madison, Wis.), New England Biolabs (Beverly, Mass.), or Applied Biosystems (Foster City, Calif.) and used according to the manufacturers' instructions. Oligonucleotide primers for sequencing and amplification of DNA were purchased from Life Technologies (Rockville, Md.). For amplification of DNA, 1 to 10 ng of template and 0.5 μM concentrations of each primer were used. All reactions included a 3-min hot start at 97°C, after which *Taq* polymerase was added and the reaction allowed to proceed for 30 to 35 cycles of denaturing (94°C for 1 min), annealing (50 to 55°C for 1 min), and primer extension (72°C for 1 min/kb of product). PCR products were purified, when necessary, by using Qiaquick cartridges (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer.

The nucleotide sequence presented below was determined either by amplifying and sequencing overlapping regions of lambda clones from a pCIM1 library or by sequencing nested deletion derivatives of subcloned DNA. The primers used were derived from the vector (T3 and T7 promoters of λ-DASHII for the

TABLE 2. Oligonucleotide primers used in this study^a

Primer	Sequence (5'-3')	<i>las</i> region coordinates	Orientation
1297	GGTGGACAGTATGAAAACAG	6575-6595	Forward
1298	GCAATCTATCTAACAATGATG	6781-6801	Reverse
1295TC	CGCAGGTATATTTTATGATTCTC	6350-6370	Forward
lasX-XF	GGGATTCCATATGAGAGTAGGAGAACTAGTAC	6382-6403	Reverse
lasX-XR	CGGGATCCACACTAGATTAGCACAGAAG	5489-5511	Forward
lasY-CF	TACCCCGGGAACAGATACTTCTTTAAAG	5648-5668	Reverse
lasY-CR	GCGCTCTAGACATATCTTTCAATGCC	4516-4523	Forward
4614	TTTCTTCCAAAGTAGAGCGGTC	4614-4635	Forward
5566	AATGTTTAAAAGCTCTTGATGGTCT	5567-5591	Forward
5751	GGAAACAATACATCTTTCTGAAATAGCTG	5724-5751	Reverse
6371	GAGAGTAGGAGAACTAGTACATCTTATTAG	6372-6401	Reverse
2476	AGAACGGGCTGTGCCATAAC	4258-4277	Reverse
2648	TTGGATGGACAGATTTCGACG	5121-5140	Reverse
2374	GTGTGGAGGAAGGTGAAATCTTG	5473-5495	Reverse
lasW-term	CTCAGTAGAAGTGGAGTTC	14955-14973	Forward
T3-DASHII	CGCGAATTAACCTCACTAAAGG	NA	NA
T7-DASHII	CCGCGTAATACGACTCACTATAG	NA	NA

^a Primers used only for sequencing were excluded. NA, not applicable.

amplified ends of inserts [Table 2]; M13 and pUC universal primers for the deletion derivatives) and a previously established pCIM1 sequence. Nested deletion derivatives of cloned DNA were generated by using the Promega Erase-a-Base kit according to the manufacturer's instructions, and plasmids intended for sequencing were purified as described previously (17). See Table 1 for details about the different clones used for completing the sequencing of the pCIM1 *las* region.

Both plasmids and amplified DNA were sequenced by using the Thermo Sequenase II (Amersham Pharmacia Biotech, Little Chalfont, England) or Big-Dye (Perkin-Elmer/Applied Biosystems) sequencing kits and the ABI Prism 377 sequencer.

Transcriptional analysis. Total RNA was isolated from *L. sakei* as described previously (38) and further purified by using RNeasy purification cartridges (Qiagen). Residual DNA was removed from the RNA preparations by DNase I (RQI; Promega) digestion, followed by RNeasy purification or phenol extraction and ethanol precipitation.

Separation and transfer of RNA for Northern analysis was carried out as described previously (38). *lasA*- and *lasX*-specific probes were generated by amplification from pCIM1 by using the primer pairs 1297-1298 and lasX-XF-lasX-XR, respectively (Table 2). After purification (Qiaquick cartridges; Qiagen) the probes were labeled by using a random priming kit (Roche Molecular Biochemicals, Basel, Switzerland) and [α -³²P]ATP (3,000 Ci/mmol; Amersham).

For the reverse transcriptase PCR (RT-PCR) analyses, first-strand cDNA synthesis was carried out according to the supplier of the RT (AMV-RT; Promega) by using 0.5 to 1.5 μ g of total RNA per reaction. Amplification of the extension products was carried out essentially as described above with a 1/10 mixture (5 μ l) of the extension reaction mix as a template and increasing the number of cycles to 40. In all cases, the forward amplification primer was identical to the one used in the corresponding RT reactions. Control reactions where RT had been omitted from otherwise identical reaction mixtures were processed simultaneously.

The primer used for identification of the *lasXY* transcript 5' end (primer 1295; see Table 2) was labeled with [γ -³²P]ATP (5,000 Ci/mmol) and polynucleotide kinase (Promega) according to the standard protocol provided by the enzyme supplier. Then 2 pmol of labeled primer was mixed with 2.5 to 5 μ g of RNA, and the volume was adjusted to 11 μ l. After a denaturing-primer-annealing step (10 min at 70°C, followed by slow [20- to 30-min] cooling to room temperature), deoxynucleoside triphosphate (50 μ M final concentration), AMV-RT buffer (1 $\times$), and enzyme (10 U) were added, and the volume was adjusted to 20 μ l. The reaction was allowed to proceed at 45°C for 60 min and was terminated by the addition of 10 μ l of stop solution-loading buffer (95% formamide, 20 mM EDTA, 0.05% each of xylene cyanol FF and bromophenol blue). After heat denaturation (70°C for 5 min), 2.5 to 5 μ l from each reaction was loaded onto a 6% polyacrylamide sequencing gel. The α -³⁵S-labeled sequencing ladders included as molecular size markers were generated with the same primer and pUC75-1 as a template in a standard Sequenase (Amersham) sequencing reaction.

Database searching, sequence retrieval, and sequence comparisons. All database searches were performed by using the basic local alignment search tool (BLAST) programs (1, 2) from the NCBI BLAST website (<http://www.ncbi.nlm.nih.gov/BLAST/>). Preliminary sequence data was obtained from The Institute of Genome Research website (<http://www.tigr.org>). Multiple alignments of proteins were generated by using CLUSTAL X (45) v.1.64b for the Macintosh.

Nucleotide accession number. The nucleotide accession number for the sequence reported in this study is Z54312.

RESULTS

Sequence of the regions flanking the *lasA-W* operon. The sequence of pCIM1 upstream of *lasA* and further downstream of *lasW* was compiled after sequencing overlapping clones from a pCIM1 lambda library and the plasmids pLas7.5-1 and pLas3.5-1 (Table 1). A graphic representation of the complete, 15,996-bp region is shown in Fig. 1.

Analysis of the sequence immediately upstream of *lasA* revealed two consecutive major ORFs, termed *lasX* and *lasY*, with an orientation opposite to the *lasA-W* operon. *lasX* encodes a 285-residue putative protein with 20 to 25% sequence identity to the members of a group of bacterial regulatory proteins. These proteins, which are usually described as Rgg-like, have been reported to regulate target gene expression by stimulating transcription initiation and have so far been found exclusively in gram-positive organisms. The target genes described include the glucosyl transferase genes in *Streptococcus gordonii* (42, 43) and *Streptococcus oralis* (8), the gene encoding a secreted cysteine protease involved in *Streptococcus pyogenes* virulence (4, 19), and genes involved in glutamate-dependent acid stress resistance in *Lactococcus lactis* (33). Recently, the involvement of an Rgg homologue in the regulation of gene expression was demonstrated in the mutacin II system of *Streptococcus mutans* (25), and a related protein is probably also involved in the expression of at least one other *Streptococcus mutans* lantibiotic, mutacin III (26). An alignment of LasX with the closest related sequences found in the databases is shown in Fig. 2.

The 300-residue putative LasY protein contains the highly conserved nucleotide binding motifs associated with the ABC

FIG. 1. The genetic organization of the *las* cluster and flanking regions. The boxed arrows represent ORFs; IS elements are shown as rectangles. The positions of transcription signals are indicated by arrows (promoters) and ball-and-stick symbols (terminators), and the positions and orientation of IS1520 (triangles and asterisks) and IS1163 insertions (triangles) are indicated.

transporter family of proteins (7, 12, 13). However, unlike the related product of *lasT*, which is expressed as part of the *lasA-W* operon and which is essential for the lactocin S phenotype (38), LasY lacks the integral domain of membrane-

spanning helices. The most similar sequences (36 to 38% identity in ungapped 288- to 289-amino-acid overlaps) identified in database searches were NatA from *Bacillus pseudofirmus* (44, 47) and *Bacillus halodurans* and YhaQ from *Bacillus subtilis*

LasX	-----MRVGELVHL IRESKNIPAINVYNSVMSKNNMFYKFEKGNFDTTSEKFMKIMSRLN	54
Rgg-Sg	--MLIVKSSGKILKIIRESKNMSLKEVAAGDISVAQLSRFERGISSLTVDSPFYSCLRNMS	58
Rgg-So	---MLETFGKIFKVIRESKMSLKEVAAGDISVAQLSRFERGVNGITLDSFYCCCKNMA	56
RopB	-----MEIGETVEFIRHSKNISIKQVCGDYLTROTYYRFIKNNLDISSKKLYILDNLN	54
MutR-II	-MLSKSLELGELYKELRMARGLKLKDIARDNLSVSQLSKFENGQTMLAADKLLATQGIH	59
MutR-III	MKVNQSMELGELYRELRIARGLKKDIACKNLSKSQLSRFENGQTMLAADKLLATSGIH	60
GadR	----MYKKYGCDFKKLRNQKNLGLSYFSKLGIDRSNISRFEHGKCMMSFERIDLMLEMO	56
LasX	ISLKEFEFFLLSEKKCLEYDTEFDKLTNAFYSKDISALQQIHNETKLMYQRTLEIR--FNN	112
Rgg-Sg	VSLAEFQYVYHNY EADDVVLQKLSBAQRENNIVKLESILAGSEAMAQEEPEKKNYKLN	118
Rgg-So	VSLEEFQYVYHNY DSDDVLFSSKQVADAYQENNVVKLQNILSSSEALTEQFEPEKKNYKLN	116
RopB	VNVDEFLFISNNFKQYKEFIDMTAKHYFECCRNIEGLNHILDSY-KDSKSTKEKN---L	109
MutR-II	MTFSEFSYAFTQY ESDLFKTGKKLVELQTKKDIKGLKKILKDY-PDTETYNVYN--RLN	116
MutR-III	MSFSEFGYALSHY ESDFFKRGHKLSELYAQKDIKGLKKLLEFN-DNHEVFDVYN--RLN	117
GadR	VPLSEYELIVNNFMPNFQEFFILELEKAEFSQNRDKIKELYSEV-----KETG---N	105
LasX	LSALSQIYYDMCSHNTTDQNTNIRNYLLNVENWYTYEWTLFNNLSFNIEDVQFLIN	172
Rgg-Sg	TIVIRATLTSCNPDYQVSKGDIETFLDYLSVEEWGRYELWLFNTVNLLETLETTFAS	178
Rgg-So	TIIVRALLSSCCSDFQISKKDIEFLTDHLYSVEEWGRYELWLFNTVDLMTLETLETTFAS	176
RopB	FALVKVLLATLTEEDCLTER--TYLSNYLINIETWSHYETVLFNNMFILESCFIEMVFS	167
MutR-II	KLVIKAAYSLDSSFEITNEEKFLTSLYLAIEEWTEYELYLFGNLFILSDDDLVFLK	176
MutR-III	KLVIQVTIHLDDTDYIISDDKNFLTLYLYNIEEWTEYELYIFNMSILSDDDLIFLKF	177
GadR	HLLTVTVTKTKLG--NIS TEVKEIEAYLCNIEEWGYFELTLEFVSDYLVNVQLELLF	162
LasX	KSLQSLSIYETHNNFGNENILVLGNIAAMYIKSYNFEKIPSLVNRMETIHLSEIAVFERL	232
Rgg-Sg	EMINRTQFYNNLPENRRRIIKMLNENVSACIENNHQVAMKFLNYIDNTKIPETDLYDRV	238
Rgg-So	EMINRTQFYNNLPENRRRIIKMLNENVISVCIEGNHLLVAMRFLNYLDHSKIPETDLYDRT	236
RopB	KVIVNLDKYNLTRYYGNESIRMFVNMLILFLORQYEDKASEILAKIEDYQLNDDCLYERC	227
MutR-II	AFVERDKLYRELSEHKKRAELVLENLILVLEHHSFYHAQYFIEKLEN-MLTYQDMFARI	235
MutR-III	AFVERDKLYISLPSHKNAELTFNLILILILERKKLYQAIYFVENLEK-LLNYQDMFAIT	236
GadR	NFDKRCENYCRVLKYRRRLLOIAYKSAIYAAGKERKKAENILEMTKKYRTVGVLDLYSEV	222
LasX	IFSEFEGVSLYVLG-TDTSLSKSLDVILKVMNMLVHLSKDHQELNIVQKNIV-----	285
Rgg-Sg	LIKYYHKAALYSYKVG-NPHARHDIQCLSTFEYLDSEFGVARKLKEQFERIQLTVVADLQIE	297
Rgg-So	LIKYYHRAALYSYKVG-NTNALSDEIQCLSFEEFLDSFGVAQK-----	276
RopB	CVSFYHRIIGLING-KEGAQKCVQILFIFQLNCKTIHMMFQTYLEAIKHKLS-----	280
MutR-II	FLNFKKKVMAAYLNG-KSTDLSDEIDYIRLVEQLGNPTLAMFLRTNLEQIMMKS-----	287
MutR-III	FLKFLKKIITYFHD-KSVDMSLEHYINIVEEI-NPTIASILKSNLQQL-----	284
GadR	LRHLARAIIFNFENAEIGEEKLYALEILEFEFGKKIKIFYQKNMKRYLKRSI-----	276

FIG. 2. Alignment (CLUSTAL X [see Materials and Methods]) of LasX with related sequences from *Streptococcus gordonii* (Rgg-Sg [42, 43]), *Streptococcus oralis* (Rgg-So [8]), *Streptococcus pyogenes* (RopB [4, 19]), *Streptococcus mutans* (MutR-II and MutR-III [25, 26]), and *Lactococcus lactis* (GadR [33]). Positions where at least four of the sequences have identical residues are highlighted (black boxes). Positions with nonidentical but similar residues are indicated with shaded boxes, and invariant positions are marked by asterisks.

(18), but significant scores were also observed for NatA from *Mycobacterium smegmatis* and for the hypothetical proteins MJ1023 and MTH1370 from the archaea *Methanococcus jannaschii* (3) and *Methanobacterium thermoautotrophicum* (41), respectively. A search of the incomplete microbial genome database (at NCBI [see Materials and Methods]) resulted in the identification of additional, related sequences in *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus anthracis*, and *Streptococcus pneumoniae*. Except for NatA from *Bacillus pseudofirmus*, which is involved in Na⁺ extrusion (47), no function has yet been assigned to any of the putative LasY homologues. Interestingly, all of the genes specifying the LasY-like proteins are closely linked to, and presumably cotranscribed with, genes encoding a homologue of the putative transmembrane protein LasW (previously Orf414 [38]).

As depicted in Fig. 1, the *las* cluster is flanked by an array of one truncated and three full-length insertion sequences belonging to three different IS families (20). Two of these elements, IS1519L and IS1519R, are nearly identical (98%), and their position and orientation (direct repeats) suggest that they may constitute the termini of a composite transposon harboring the *las* gene cluster. IS1519L and IS1519R belong to the IS6 family of IS elements, which typically generate 8-bp direct repeats of the target sequence upon transposition (20). In the case of pCIM1, such repeats were not found, neither flanking each element nor associated with the IS1519L-*las* cluster-IS1519R unit. The absence of direct repeats was also noted for IS1516, which is an ISL3-type element situated ca. 350 and 450 bp from *lasY* and IS1519L, respectively. IS1516 is probably nonfunctional, since the putative transposase gene has a frameshift after codon 151. The 267 bp immediately distal (with respect to the *las*-region, see Fig. 1) to IS1519L shows significant similarity to the right end of elements belonging to the IS30 family of insertion sequences, and this truncated IS element has been designated IS1517.

The leftmost 1,200 bp (according to the orientation shown in Fig. 1) of the sequenced region contain three ORFs that could be involved in the replication or maintenance of pCIM1: the translation of *orf78* is 40% identical to the C terminus of RepB, which is a replication-associated protein of the *Enterococcus faecalis* plasmid pAD1 (46). *orf116*, which overlaps the 3' end of *orf78* by 5 bp, encodes a protein similar (25% identical) to the 123-residue protein RepB implicated in stable maintenance of the *Lactobacillus reuteri* plasmid pTE15 (C. F. Lin and T. C. Chung, unpublished data; GenBank accession no. AF036766). The third ORF in this region, *orf130*, overlaps the proposed left inverted repeat of the truncated insertion sequence IS1517 by 6 bp. The translation of *orf130* is 32% identical (over a 100-residue region containing one gap) to the C terminus of FipA, which is a pKM101-encoded inhibitor of IncP plasmid transfer functions (34).

Transcription analysis. The similarity to Rgg and related proteins suggested that LasX might be a transcriptional regulator of lactocin S expression. In order to assay the effect of *lasX* inactivation on the transcription of the *lasA-W* operon, RNA from L45 and the *lasX* insertion mutants was analyzed by Northern hybridization by using a *lasA*-specific probe. As shown in Fig. 3A, the three mutants tested had a strongly reduced level of *lasA*-specific RNA compared to the wild type. The level of *lasA*-specific mRNA was not significantly affected

FIG. 3. Northern blots of RNA isolated from *L. sakei* L45 wt and mutants hybridized with a *lasA*-specific probe (see Materials and Methods and Table 1). (A) Lanes 1 and 6, L45-2.1 (wild type); lanes 2 to 4, *lasX* mutants L45-5.3, L45-5.6, and L45-5.7, respectively; lane 5, L45-3.1 (Bac⁻Imm⁻). (B) Lane 1, L45-2.1; lane 2, L45-3.1; lane 3, L45-5.1(*lasM*::IS1163); lane 4, L45-5.2(*lasT*::IS1163); lane 5, L45-5.7(*lasX*::IS1520); lane 6, L45-5.8 (*lasN*::IS1520).

in any of the *lasT*, *lasM*, or *lasN* mutants tested (Fig. 3B). All of the mutations represented in Fig. 3B are nonpolar, as determined by Northern hybridization (38) or RT-PCR of downstream sequences (40).

The close spacing (70 bp) between *lasX* and *lasY* and the absence of likely promoter sequences upstream of *lasY* suggests that the two genes may be cotranscribed, an assumption which was initially tested by carrying out hybridization experiments like the one described above. However, this approach failed to detect *lasX* and *lasXY* transcripts in any of the RNA preparations, indicating a low level of transcription and/or rapid degradation of the transcript(s). Therefore, a series of RT-PCR experiments were carried out with combinations of primers derived from the ends and interior of both ORFs. The results of these experiments (Fig. 4) demonstrate the presence of mRNA representing both genes, thereby supporting the notion that *lasX* and *lasY* are expressed as an operon.

Identification of the 5' end of the *lasXY* mRNA. The initiation codons of the divergently oriented *lasA* and *lasX* ORFs are situated only 182 bp apart, and transcription of the *lasA-W* unit initiates ca. 110 bp upstream of *lasA*. This condensed organization suggested some degree of overlap between the promoter of the *lasXY* unit, P_{XY}, and P_{A-W}. The transcription start of the *lasX* or *lasXY* mRNA was identified (see Materials and Methods) by using RNA isolated from late-growth-phase cultures and a primer complementary to nucleotides 32 to 54 of the *lasX* coding sequence (*las* region coordinates 6350 to 6372 [see Table 2]). The results of the primer extension experiment (Fig. 5) indicate the C, located 36 nucleotides upstream of the coding sequence, as the 5'-terminal residue of the *lasXY* transcript. As shown in Fig. 5, the distance between the 5' ends of the divergent *lasXY* and *lasA-W* transcripts is only 34 bp, and both of the suggested -35 regions mask the initiation point of

FIG. 4. RT-PCR detection of *lasX* and *lasY* transcripts. The approximate positions of the primers used (see Table 2) are indicated at the top. The reactions were performed with L45–2.1 RNA and primers 5566 (first-strand cDNA synthesis and PCR) and 6371 (lanes 1 and 2) or primers 4614 (first-strand cDNA and PCR) and 5751 (lanes 3 and 4). As a control of RNA-dependent amplification, RT was omitted from the samples run in lanes 2 and 4. The 5566–6371 and 4614–5751 PCR products with pCIM1 DNA as a template were run as molecular size controls in lanes 5 and 6, respectively.

the oppositely directed transcript. This organization of regulator and target promoters is unique among the Rgg-type regulons.

DISCUSSION

We report here the results of the continued sequencing of the lactocin S-encoding region of pCIM1, an effort spurred by the identification of *las* insertion mutations mapping outside of the previously reported lactocin S gene cluster (40). Two consecutive ORFs, termed *lasX* and *lasY*, were identified immediately upstream of the P_{A-W} promoter. Transcription analysis indicates that *lasX* and *lasY* are cotranscribed, thereby constituting a second lactocin S operon. The translation of *lasX*, which is the affected gene in all of the new mutants, is similar to the streptococcal Rgg (42, 43) and other proteins shown to be involved in the regulation of gene expression in gram-positive bacteria. The observed correlation between the Bac[−] phenotype and the level of *lasA*-specific mRNA in *lasX* mutants suggests that the product of *lasX* regulates lactocin S gene expression at the transcription level. These findings agree well with what has been observed for Rgg and its homologs in other systems, where it has been concluded that they function as transcriptional activators (4, 19, 25, 42, 43).

Given the similarity of the sequence, as well as of apparent function, it seems reasonable to assume that the Rgg-type regulators may also have a common mechanism of target gene activation. Positive regulation of prokaryotic gene expression often involves the binding of transcription factor molecules to

FIG. 5. On the left-hand side is shown a representation of the *las* cluster regulatory region. The features indicated are the experimentally determined transcription initiation points (bent arrows), the deduced promoter elements (underlined), direct repeats (marked DR1 to DR3 [with brackets]), and the proposed ribosome-binding sites (boldface letters). The roman numerals refer to the 5' ends of the two RNA species representing *lasA* (38). To the right is shown the result of the primer extension experiment performed to determine the 5' end of *lasX* (and/or *lasXY*) mRNA. The arrow indicates the extension product of the primer 1295TC (Table 2), which was run alongside a sequence reaction with 1295TC and plas7.5–1 as a template.

short, specific sequence motifs in the vicinity of the target promoter (14). In the lactocin S promoter region, the most conspicuous candidates for *cis*-acting regulatory elements are two directly repeated heptanucleotide motifs (TTATCCC) located in the region from -40 to -70 (relative to the A/A-W transcript 5' end), with a third, imperfect unit (TTaCCC) overlapping the -10 promoter element. The position and spacing of the direct repeats is similar to the corresponding features in the otherwise unrelated phosphorelay systems regulating bacteriocin production in several lactic acid bacteria (for a recent review, see reference 24). It is tempting to speculate that the proposed stimulation of transcription from the P_{lasA-W} promoter is triggered by the binding of LasX to the upstream repeats. The overlapping, divergently oriented organization of regulator and target promoters suggests that interdependent expression of the two *las* operons may occur by steric interaction alone. In addition, the condensed organization of the *lasX-lasA* intergenic region (Fig. 5) points to the interesting possibility that the putative protein-DNA interaction promoting transcription from P_{A-W} could, simultaneously, specifically downregulate transcription from P_{XY} .

Putative *cis*-acting elements have been identified in other Rgg-regulons also, but there is a notable lack of structural similarities between the different target promoter regions. In the absence of experimental data, therefore, one has to consider the possibility that the transcription-promoting interaction between Rgg-type proteins and RNA polymerase takes place without prior binding of the transcription factor to the promoter region (14).

We are currently investigating in more detail, by techniques such as quantitative RT-PCR and band-shift analysis, the interaction between LasX and its suspected recognition sites in the *lasX-lasA* intergenic region. Hopefully, the results of these efforts will contribute to a better understanding of how the lactocin S and, possibly, related regulons work.

The *lasA-W* and *lasXY* operons comprise a tightly clustered, 11 ORF unit, which is punctuated by an insertion sequence(s) on both sides. At face value, the organization of the nearly identical elements IS1519L and IS1519R indicates that they may constitute the termini of a compound transposon carrying a second IS element, as well as the genes specifying the lactocin S phenotype. Similar arrangements have been observed in several cases for the nisin (10, 15, 16, 27, 28, 36) and, more recently, for the lactacin 481 (5) gene clusters. The absence of direct repeats flanking the IS1519 elements weakens the hypothesis of a lactocin S transposon. It should be noted, however, that transposition by the IS6 type insertion sequences generally lead to replicon fusions or cointegrates (9, 20), in which the fused replicons are separated by directly repeated copies of the IS element. It is possible that the lactocin S region initially was generated by an IS1519-mediated cointegrate formation and that the present locus is the result of a subsequent homologous recombination reaction(s) involving one or both of the flanking repeats. Since there are no additional copies of IS1519 in the *L. sakei* L45 genome, such (hypothetical) events must have occurred prior to the establishment of the lactocin S gene cluster in L45.

Although the organization of the two *las* operons suggests that they constitute a complete locus, we cannot at this point exclude the existence of additional, specific genes involved in

the production of lactocin S. It is also still an open question whether all, or just a subset of the genes within the cluster are necessary for production, since many of the ORFs have no obvious function in determining the lactocin S phenotype. These and related issues are currently being investigated in our laboratory by heterologous expression experiments in conjunction with systematic mutational analysis of the complete region.

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