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Characterization of the Lacticin 481 Operon: the *Lactococcus lactis* Genes *lctF*, *lctE*, and *lctG* Encode a Putative ABC Transporter Involved in Bacteriocin Immunity

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The lantibiotic lacticin 481 is a bacteriocin produced by *Lactococcus lactis* strains. The genetic determinants of lacticin 481 production are organized as an operon encoded by a 70-kb plasmid. We previously reported the first three genes of this operon, *lctA*, *lctM*, and *lctT*, which are involved in the bacteriocin biosynthesis and export (A. Rincé, A. Dufour, S. Le Pogam, D. Thuault, C. M. Bourgeois, and J.-P. Le Pennec, Appl. Environ. Microbiol. 60:1652–1657, 1994). The operon contains three additional open reading frames: *lctF*, *lctE*, and *lctG*. The hydrophobicity profiles and sequence similarities strongly suggest that the three gene products associate to form an ABC transporter. When the three genes were coexpressed into a lacticin 481-sensitive *L. lactis* strain, the strain became resistant to the bacteriocin. This protection could not be obtained when any of the three genes was deleted, confirming that *lctF*, *lctE*, and *lctG* are all necessary to provide immunity to lacticin 481. The quantification of the levels of immunity showed that *lctF*, *lctE*, and *lctG* could account for at least 6% and up to 100% of the immunity of the wild-type lacticin 481 producer strain, depending on the gene expression regulation. The lacticin 481 biosynthesis and immunity systems are discussed and compared to other lantibiotic systems.

Great emphasis has been placed in the past few years on the isolation and study of bacteriocins. These compounds are proteinaceous bactericidal molecules produced by a wide range of microorganisms (17). Bacteriocins produced by food-grade organisms like lactic acid bacteria are of particular interest, due to their potential application in food preservation. Lacticin 481 is such a bacteriocin, being produced by *Lactococcus lactis* strains and including in its spectrum of inhibitory activity *Clostridium tyrobutyricum*, responsible for late swelling of Emmental-type cheese (43). Four classes of bacteriocins from gram-positive bacteria have been distinguished (22), and lacticin 481 is a member of the lantibiotic family (class I) (27). Lantibiotics are characterized by the presence of the rare amino acids lanthionine, β-methyl lanthionine, and dehydrated residues (18). Like the other lantibiotics, lacticin 481 is ribosomally synthesized as a prepropeptide. The lacticin 481 prepropeptide consists of an N-terminal leader peptide of 24 residues followed by a 27-residue propeptide moiety (27, 28). Lacticin 481 is excreted after posttranslational modifications of the propeptide and cleavage of the leader sequence. Mature lacticin 481 contains three thioether bridges connecting residues 9 and 14, 11 and 25, and 18 and 26 (45). The bactericidal action of lantibiotics is generally caused by the formation of voltage-dependent short-lived pores, which causes an efflux of ions and small molecules (1, 17, 32). A recent study supports a similar mode of action for lacticin 481 (6).

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According to the structure imposed by the thioether rings of lanthionine residues, lantibiotics can be grouped into linear (type A) and globular (type B) peptides (18). Within group A lantibiotics, homologies of the prepropeptide sequences and location of the modified residues allowed Sahl et al. to distinguish three subgroups (33). The nisin subgroup includes nisin, subtilin, epidermin, Pep5, and epilancin K7, whereas the lacticin 481 subgroup contains lacticin 481, streptococcin A-FF22, salivaricin A, streptococcin A-M49, and variacin (16, 30, 33). Bacteriocins from these two subgroups are distinct in respect to both their ring structure and their leader peptide with a characteristic cleavage site (13, 33, 45). Lactocin S and cytolysin, which have leader peptides showing some similarities with those of the lacticin 481 subgroup (13) but unrelated propeptide parts, were classified in a third subgroup (33). (It should be noted that de Vos et al. distinguished only two subgroups of linear lantibiotics: class AI, which corresponds to the nisin subgroup, and class AII, regrouping the other linear lantibiotics, including lacticin 481 [7].)

A number of lantibiotic gene clusters are well characterized, especially for bacteriocins of the nisin subgroup and, to a lesser extent, of the lactocin S subgroup (38). These clusters include the lantibiotic structural gene plus genes for bacteriocin expression, modification, transport, and immunity and, in some cases, regulatory genes. In the lacticin 481 subgroup, apart from lacticin 481 itself, only the lantibiotic structural genes have been described so far. Lacticin 481 from two *L. lactis* strains of distinct origins, CNRZ 481 and ADRIA 85LO30 (26, 43), has been studied independently. Piard et al. described *lctA*, the structural gene for lacticin 481, cloned from *L. lactis* CNRZ 481 (27). The genetic determinants of the *L. lactis* ADRIA 85LO30 bacteriocin, which was named lactococcin DR, are organized as an operon located on a 70-kb plasmid (9), and we previously reported the first three genes of this

operon: *lcnDR1*, *-2*, and *-3* (31). *lcnDR1* is identical to *lctA* and is thus the lacticin 481 structural gene. Therefore, lactococcin DR and *lcnDR1*, *-2*, and *-3* have been renamed lacticin 481, *lctA*, *lctM*, and *lctT*, respectively. *lctM* is essential for lacticin 481 biosynthesis in *L. lactis* IL1403 (31). LctM has significant homologies with LasM and CylM, deduced from genes of the lactocin S and cytolysin gene clusters, respectively (11, 40), suggesting that LctM is involved in serine and threonine dehydration reactions and in ring formations (38). The LctT dimer forms an ABC transporter which has the dual function of cleaving the leader peptide and excreting mature lacticin 481 (12).

Here we report the cloning, sequencing, and expression of the *lctF*, *lctE*, and *lctG* genes, which are located downstream of *lctT* in the lacticin 481 operon. The proteins encoded by these three genes belong to the ABC transporter family and confer immunity to lacticin 481. The lacticin 481 operon does not seem to include an additional gene(s) and is thus the first-characterized operon of the lacticin 481 subgroup. A comparison is drawn with gene clusters of bacteriocins from the other type A lantibiotic subgroups.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The lacticin 481-sensitive and nonproducing *L. lactis* strains were IL1403 (plasmid free) (5) and IL1835 [IL1403 harboring the vector pIL252 (Em^r)] (39). The wild-type strain producing lacticin 481 was *L. lactis* ADRIA 85LO30 (43). All *L. lactis* strains were grown in GM17 (42). *Escherichia coli* TG2 (34) was used for plasmid constructions and amplifications. Recombinant phages were selected and propagated in *E. coli* Q359 and Q358, respectively (19). Antibiotics were ampicillin (100 $\mu g \cdot ml^{-1}$), erythromycin (300 $\mu g \cdot ml^{-1}$), and kanamycin (10 $\mu g \cdot ml^{-1}$) for *E. coli* and erythromycin (5 $\mu g \cdot ml^{-1}$) for *L. lactis.*

Production and immunity assays. Inhibitory activity was assayed by either the culture supernatant diffusion method or the solid agar medium test, as previously described (9), the indicator strain being *L. lactis* IL1835 (39). To quantify lacticin 481, twofold bacteriocin dilutions were tested by the supernatant diffusion method. The number of arbitrary units (AU) was the dilution factor which produced a 1-mm inhibitory halo versus IL1835. When a higher lacticin 481 concentration was requisite, supernatants of *L. lactis* ADRIA 85LO30 cultures were concentrated up to 16-fold by dialysis versus solid polyethylene glycol 8000 for 48 h at 4°C, in dialysis membranes with a molecular mass cutoff of 500 Da. Bacteriocin immunity assays were performed according to the culture supernatant diffusion method, after a GM17 agar (15 $g \cdot liter^{-1}$) plate was seeded with a 6-h culture of the test strain (0.03 unit of optical density at 570 nm).

General methods. Plasmids were extracted from *L. lactis* strains as described by Anderson and McKay (3). Plasmid preparations from *E. coli* were obtained and DNA manipulations were performed according to the methods of Sambrook et al. (34). *L. lactis* and *E. coli* were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories) as described by Holo and Nes (15) and Dower et al. (8), respectively. RNAs were isolated as described by Keilhauer et al. (21). Dot blot analyses were performed with nylon membranes (Hybond-N^+; Amersham) according to the standard protocol recommended by the membranes' supplier.

Sequence analysis. Nucleotide sequencing was carried out on double-stranded DNA with the T7 sequencing kit (Pharmacia) and the Sequenase kit (Amersham). The complete nucleotide sequence was determined on both strands from exonuclease III deletion fragments and subcloned DNA fragments. Synthetic 20-mer oligonucleotides were used as primers when necessary. The open reading frames (ORFs) were determined with the DNA program (36). The hydrophobicity profiles were obtained by the method of Kyte and Doolittle (24). Similarity searches with nonredundant database proteins were performed with the BLAST program (2).

Plasmid constructions. The lactococcal plasmids pIL253 (high copy number, Em^r) and pIL252 (low copy number, Em^r) (39), the *E. coli* plasmid pBluescript (Amp^r) (34), and the *E. coli* bacteriophage λ2001 (20) were used as cloning vectors. The vector part of pEF constructs is pBluescript, whereas pEB constructs include both pBluescript and pIL253 or pIL252.

The lacticin 481 genetic determinants are included in the del10 DNA region from pOS5 (9, 31). The del10 region was originally defined by three contiguous *Bam*HI fragments (Fig. 1A): B1-B2 (2.1 kb), B2-B3 (4.5 kb), and B3-B4 (3.1 kb) (9). They were cloned into pBluescript, and recombinant plasmids containing B2-B3 and B3-B4 were named pEF75 and pEF88, respectively (Fig. 1C). pEF84 and pEF92 were derived from these plasmids (Fig. 1C). λ2001 was used to clone from pES2, a pOS5 variant (9), a 10-kb *Bcl*I fragment extending from a site next to B2 to a site downstream of B4 (phage λC40 [Fig. 1C]). The *Xba*I fragment X4-X5 was subcloned into pBluescript from λC40, creating pEF90 (Fig. 1C). pEF57 is composed of pBluescript and the insert of pEB57 (Fig. 1C) (31). pEF-kP was constructed by subcloning the following into pBluescript: first, a 0.1-kb *Eco*RI-*Bam*HI DNA fragment containing the strong constitutive lactococcal promoter P32 from pSI19 (10) and then, upstream of P32, a *Hin*dIII fragment containing the kanamycin resistance cassette (*kanR* gene) from p17635 (4). The *kanR* gene facilitated the selection of transformants harboring the expected constructs in the course of the subsequent cloning experiments.

The strategy used to place the *lctF*, *lctE*, and *lctG* genes under the control of a lactococcal promoter was the following. The *kanR* gene and the P32 promoter were excised from pEF-kP as a single 1.7-kb fragment by *Sac*I digestion. This fragment was subcloned in pEF84, upstream of the *Xba*I site X4 (Fig. 1C). *E. coli* transformants were selected with both ampicillin and kanamycin, and the resulting plasmid, pEF-F′ (Fig. 1D), contains in the appropriate orientation *kanR*, P32, the 3′ end of *lctT*, and the 5′ end of *lctF*. The *kanR* gene from p17635 was also cloned independently into the *Nco*I site of pEF75 (Fig. 1C), to produce pEF75-k1. The insert of the latter was purified and subcloned into the *Bam*HI site B3 of pEF92 (Fig. 1C), leading to pEF93-k1 (Fig. 1D). A ligation between the 3.8-kb *Sca*I DNA fragment from pEF-F′ and the 3.9-kb *Sca*I fragment from pEF93-k1 generated pEF-FEG (Fig. 1D), which contains *lctF*, *lctE*, and *lctG* downstream of the P32 promoter. *E. coli* transformants harboring the pEF-FEG recombinant plasmid were selected with both ampicillin and kanamycin, and the junctions between the different cloned fragments were verified by sequencing. From pEF-FEG, deletions were constructed in *lctF* or *lctE* as follows (Fig. 1D). The *lctF* deletion plasmid pEF-EG was obtained by elimination of the *Bam*HI-B3 fragment of pEF-FEG. To obtain an *lctE* deletion (pEF-FG), the *Acy*I-*Sty*I fragment was eliminated from pEF-FEG. To be able to transfer the constructs into a lactococcal strain, the inserts of pEF-FEG and pEF-EG were purified after *Apa*I digestion and subcloned into the *Apa*I site of pBS-pIL, which is a fusion of the two vectors pBluescript and pIL253. The recombinant plasmids, pEB-FEG and pEB-EG, respectively, were obtained after electroporation of *E. coli* and double selection with kanamycin and erythromycin. pIL253 was fused to pEF-FG after linearization of both plasmids by *Sma*I, creating pEB-FG.

Reconstruction of the complete lacticin 481 operon was done as follows. The 6.6-kb *Apa*I fragment of pEF93-k1 was ligated with pEF57 linearized with *Apa*I (Fig. 1D). The *Nco*I fragment of the resulting construct pEF94-k1 was deleted to create pEF94 (Fig. 1C and D). The junction sites between the different fragments were confirmed by DNA sequencing. To be able to introduce the construct into *L. lactis*, pIL253 was fused to pEF94 after linearization of the two plasmids by *Xho*I, creating pEB94. To obtain a low-copy-number version of pEB94, pEF94 was also fused to pIL252, leading to pEB170.

To construct plasmids expressing *lctF*, *lctE*, and *lctG* from the promoters of the lacticin 481 operon, the *Sna*BI fragment was deleted from pEF94, creating pEF102 (Fig. 1C). pEB118 and pEB150 were constructed by fusing pIL253 and pIL252, respectively, to pEF102, after linearization of the plasmids by *Xho*I. An *lctG* deletion was obtained from pEF102 by deleting the E3-*Cla*I fragment, *Cla*I being part of the pBluescript polylinker. The resulting plasmid, pEF106 (Fig. 1C), was fused to pIL253 at the *Xho*I site, creating pEB126.

Nucleotide sequence accession number. The DNA sequence has been assigned GenBank accession no. U91581.

RESULTS

Nucleotide sequence of the 3′ end of the lacticin 481 operon. We previously localized the lacticin 481 genetic determinants on a 10-kb DNA region (named del10) from pOS5, a 70-kb plasmid (9). The lacticin 481 structural gene was identified as the first gene (*lctA*) of an operon including at least two other genes, *lctM* and *lctT* (31). In order to fully characterize this operon, the nucleotide sequence was extended to the region downstream of *lctT*. pEF88 and pEF90 (Fig. 1C) were constructed as described in Materials and Methods, and their inserts were sequenced on both strands. Within the 3,656 bp newly sequenced, three complete ORFs were identified and named *lctF*, *lctE*, and *lctG* on the basis of the similarities described below. *lctF*, *lctE*, and *lctG* potentially code for proteins of 306, 250, and 245 residues with calculated molecular masses of 34.4, 29.2, and 28.4 kDa, respectively. The beginning of an additional ORF longer than 309 codons and named *orf7* was located downstream of *lctG*. All these ORFs are in the same transcriptional direction. An 11-bp inverted repeat typical of a Rho-independent terminator was found between *lctG* and *orf7*. This terminator is followed by a putative promoter with the −35 and −10 sequences TTGATA and AATAAA, respectively, separated by 16 bp. *orf7* is thus unlikely to belong to the lacticin 481 transcription unit. *lctF*, *lctE*, and *lctG* are contiguous since the initiation codon of *lctE* overlaps the *lctF*

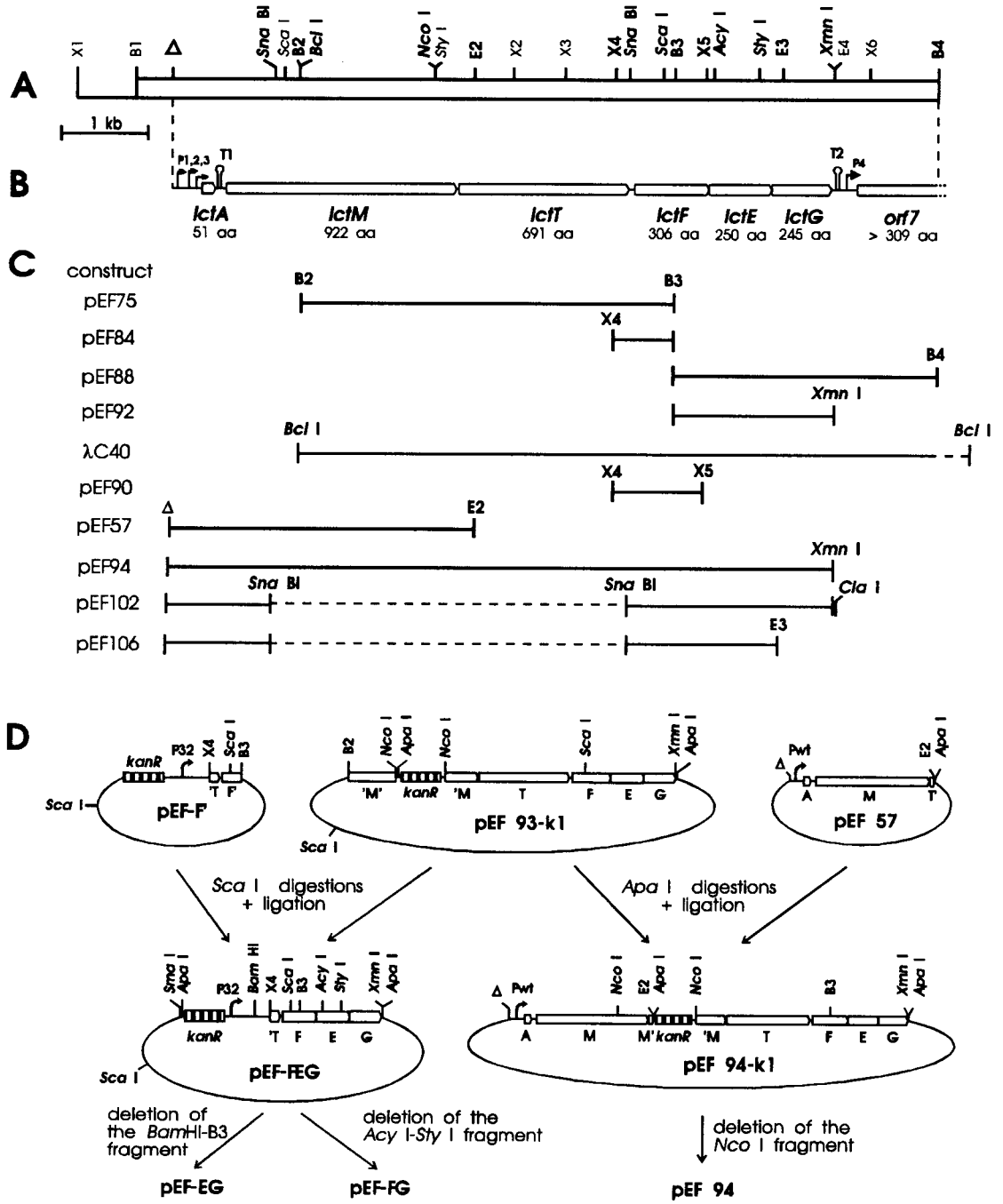

FIG. 1. (A) Partial restriction enzyme map of the del10 region of pOS5 including lacticin 481 genetic determinants. The open box indicates the del10 region. *Eco*RI (E), *Bam*HI (B), and *Xba*I (X) sites are numbered in order to permit unambiguous designation of the DNA restriction fragments in the text. Δ indicates the first base of the previously published sequence (31). Sites in boldface are those used in the cloning experiments. (B) Genetic organization of the lacticin 481 operon. The open boxes represent the ORFs, and their orientation shows the transcriptional direction. The amino acid (aa) lengths of the encoded proteins are indicated. The genes of the lacticin 481 operon are the *lct* genes, and an additional gene is named *orf7*. P1 and P3 are functional promoters (31), P2 and P4 are putative promoters, and T1 and T2 are putative Rho-independent terminators. (C) Fragments of del10 inserted into the indicated constructs. pBluescript is the vector of all the pEF plasmids, whereas the phage λC40 is based on the λ2001 vector. Dashed lines represent deleted DNA fragments. (D) Strategies used for the construction of pEF-FEG, pEF-EG, pEF-FG, and pEF94. The boxes represent the ORFs. *lctA*, *lctM*, *lctT*, *lctF*, *lctE*, and *lctG* are designated A, M, T, F, E, and G, respectively. Names of ORFs are preceded or followed by an apostrophe when their 5′ or 3′ end is missing. *kanR*, kanamycin resistance gene. Pwt, the three potential wild-type promoters P1, P2, and P3 of the lacticin 481 operon. P32, constitutive lactococcal promoter. Cloning details are described in Materials and Methods.

stop codon and the first codon of *lctG* overlaps the last two codons of *lctE*. *lctF* starts 56 bp downstream of the *lctT* stop codon, but no potential terminator sequence was found between these two ORFs. We also extended our sequence upstream of *lctA* by 700 bp and found the 3′ end of a gene whose product is homologous to transposases (data not shown) and thus very probably unrelated to bacteriocin production. The same gene had already been found by Piard et al. upstream of

the *lctA* gene from a different *L. lactis* strain (27). The lacticin 481 operon is thus proposed to include a total of six genes: *lctA*, *lctM*, *lctT*, *lctF*, *lctE*, and *lctG* (Fig. 1B).

LctF, LctE, and LctG are similar to ABC transporters. The screening of protein databases revealed similarities between LctF, LctE, and LctG and proteins forming typical ABC transporters. ABC transporters are transmembrane transporters showing a relative specificity for a given substrate. Transport energy is provided by ATP hydrolysis, and the most characteristic feature of these transporters is a highly conserved ATP binding cassette. A typical ABC transporter is composed of four domains: two of them are highly hydrophobic and each consists of six membrane-spanning segments, whereas the other two are the ATP binding domains, peripherally located at the internal face of the membrane. Each ATP binding domain includes the Walker motifs A and B. The four domains can be expressed as separate polypeptides or fused into larger, multifunctional polypeptides (14). For example, LctT would be a fusion of one transmembrane domain and one ATP binding domain (plus one protease domain) and would form a complete ABC transporter after dimerization (12).

LctF showed its strongest sequence identities with EpiF (25), BcrA (29), and NisF (37) and with the N-terminal region of SpaF (23). Identities of 52.0, 41.8, 41.3, and 40.5% were found between these respective proteins and LctF (Fig. 2A). Like these proteins, LctF possesses the two Walker motifs A and B (Fig. 2A). BcrA provides a mechanism of resistance to the peptidic antibiotic bacitracin (29). SpaF, NisF, and EpiF are involved in the immunity to the lantibiotics subtilin, nisin, and epidermin, respectively (23, 25, 37). Hydrophobicity profiles revealed that LctF is hydrophilic, like BcrA, NisF, EpiF, and the N-terminal part of SpaF (data not shown).

The 250- and 245-amino-acid LctE and LctG are similar to each other (24.8% identity and 61.1% similarity) (Fig. 2B). Their hydrophobicity profiles are well conserved, with predicted hydrophobic structures forming six potential membrane-spanning domains (Fig. 2C). These proteins showed significant sequence similarities with the C-terminal domain of SpaF and with SpaG, NisE, NisG, EpiE, and EpiG, which are involved in immunity to the lantibiotics subtilin (SpaF and SpaG), nisin (NisE and NisG), and epidermin (EpiE and EpiG) (23, 25, 37). All these proteins exhibited similar hydrophobicity profiles. The highest scores were obtained between LctE and EpiE and between LctG and NisG (28.0 and 21.0% identity, respectively).

No significant similarity was found to the 309 N-terminal residues deduced from *orf7*.

Coexpression of *lctF*, *lctE*, and *lctG* confers immunity to lacticin 481. The similarities described above prompted us to examine whether expressing *lctF*, *lctE*, and *lctG* would provide a lacticin 481 immunity phenotype for an *L. lactis* strain. As described in Materials and Methods, several constructs were created to place all three genes (pEB-FEG) (Fig. 1D) or combinations of two of them (pEB-EG and pEB-FG containing *lctE* and *lctG* and *lctF* and *lctG*, respectively) in a lactococcal vector and under the control of a strong constitutive lactococcal promoter, P32. pEB-FEG, pEB-EG, and pEB-FG were introduced into *L. lactis* IL1403, and the resulting strains were designated IL-FEG, IL-EG, and IL-FG, respectively. As a negative control, the insert of pEF-kP (*kanR* and P32) was subcloned into pBS-pIL, a fusion of pBluescript and pIL253, creating pEB-kP and the strain IL-kP when IL1403 was transformed by the latter.

To test the immunity to lacticin 481, the supernatant of an *L. lactis* ADRIA 85LO30 culture was used as a bacteriocin source and loaded into the well of an agar plate previously inoculated with the test strain. After growth of the strain, an inhibitory halo around the well was observed if the strain was sensitive to lacticin 481, as illustrated by *L. lactis* IL-kP (Fig. 3A, panel 1). In contrast, no halo appeared if the test strain was immune, like the bacteriocin producer ADRIA 85LO30 itself (Fig. 3A, panel 5). No growth inhibitory halo was observed with IL-FEG (Fig. 3A, panel 2), showing that coexpression of *lctF*, *lctE*, and *lctG* was providing a phenotype of immunity to lacticin 481. In contrast, in testing IL-EG or IL-FG, we observed a halo as large as that seen with IL-kP (Fig. 3A, panels 3 and 4). As a control for gene expression and RNA stability, *lctG* mRNA accumulation was analyzed by dot blot. Signals of similar strength were obtained with RNA preparations from IL-FEG, IL-EG, and IL-FG (Fig. 3B, panels 2 to 4), whereas no signal was detected from IL-kP (Fig. 3B, panel 1). The nonimmune phenotype of IL-EG or IL-FG is thus due not to a lack of expression of the genes, but rather to the deletions created in *lctF* or *lctE*. Both *lctF* and *lctE* are therefore necessary to confer on an *L. lactis* strain immunity against lacticin 481.

Despite several trials using various strategies, we were unable to construct in *E. coli* a plasmid expressing only *lctF* and *lctE* under the control of P32. The latter being a strong and constitutive promoter, the level of *lctF* and *lctE* expression achieved under its control could be toxic to the bacteria in absence of LctG. We therefore tried to place *lctF*, *lctE*, and *lctG* under their wild-type expression control system (i.e., the promoters P1, P2, and P3 and the terminator T1 found upstream and downstream of *lctA*, respectively [Fig. 1B]), rather than under P32 control. In the resulting plasmid, pEF102, which includes *lctA*, *lctF*, *lctE*, and *lctG* (Fig. 1C), the level of transcription of *lctF*, *lctE*, and *lctG* is expected to be lower than that in pEF-FEG, especially because of the terminator sequence, which is proposed to act as an attenuator (see Discussion). When pEF102 was fused to the lactococcal vector pIL253 and introduced into *L. lactis* IL1403, the strain became resistant to lacticin 481 (data not shown), showing that the transcription level of the three genes was high enough to provide lacticin 481 immunity. From pEF102, it was possible to delete *lctG* (pEF106, Fig. 1C), probably because the transcription level of *lctF* and *lctE* was too low to result in a toxic accumulation of LctF and LctE. When *L. lactis* IL1403 was transformed by pEF106 fused to pIL253, the strain remained as sensitive to lacticin 481 as IL-EG and IL-FG (data not shown). *lctG* is thus necessary, like *lctF* and *lctE*, to obtain the lacticin 481 immunity phenotype.

Cloning and expression of the complete lacticin 481 operon in *L. lactis* IL1403. As the lacticin 481 operon had never been cloned on a single fragment, we reconstructed the complete operon and introduced it into *L. lactis* in order to verify whether the resulting bacteria are able to produce the lantibiotic and are immune to it. The plasmid pEB94, containing the entire operon controlled by its own promoters, was constructed as described in Materials and Methods. For comparison, we also used pEB57, which allows the expression of the first two genes, *lctA* and *lctM* (31). For subsequent experiments, these two plasmids were electroporated into *L. lactis* IL1403, creating strains IL94 and IL57.

Growth of the indicator strain IL1835 was not inhibited by IL1403, containing the vector pBS-pIL, a fusion of pBluescript and pIL253 (Fig. 4A, panel 1). As we already noticed (31), a small inhibitory halo was observed when the first two genes of the operon were introduced into IL1403 (Fig. 4A, panel 2). The halos were larger around colonies of IL94 expressing the entire operon (Fig. 4A, panel 3). Halos obtained with *L. lactis* ADRIA 85LO30 were larger than those with IL94 (Fig. 4A, panel 4), showing that the wild-type strain is a more effective

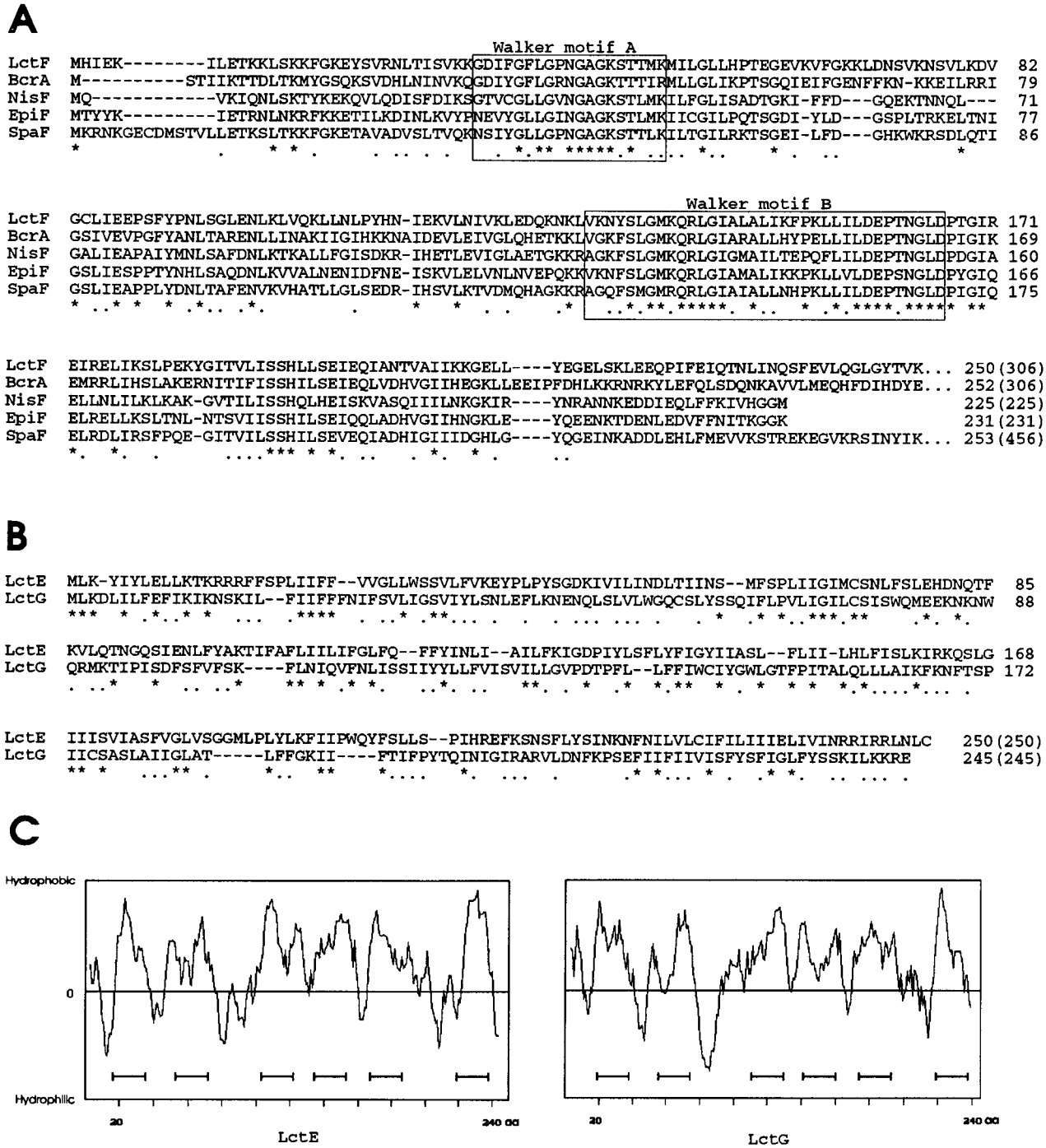

FIG. 2. (A) Similarities among LctF, BcrA (29), NisF (37), EpiF (25), and the N-terminal part of SpaF (23). Residues conserved in all five proteins are indicated by asterisks. Similar residues are shown by dots. Boxes contain the Walker motifs A and B. The amino acids are numbered, and the total length of each protein is indicated in parentheses. (B) Similarities between LctE and LctG. Asterisks and dots show identical and similar residues, respectively. (C) Hydrophobicity profiles of LctE and LctG, determined according to the method of Kyte and Doolittle (24). Bars indicate the hydrophobic segments proposed to be membrane spanning.

lacticin 481 producer than IL1403 harboring the entire lacticin 481 operon.

When the immunity of the four strains was tested, both ADRIA 85LO30 and IL94 were immune to lacticin 481 (Fig. 4B, panels 3 and 4), while IL57 or IL1403 containing just the vector was sensitive to the bacteriocin (Fig. 4B, panels 1 and 2).

Quantification of the level of immunity conferred by *lctF*, *lctE*, and *lctG*. We examined whether *lctF*, *lctE*, and *lctG* could be sufficient to account for the immunity of *L. lactis* ADRIA 85LO30, the wild-type lacticin 481 producer strain. As described in Materials and Methods, lacticin 481 was concentrated to obtain a final concentration of up to 1280 AU · ml^{-1}.

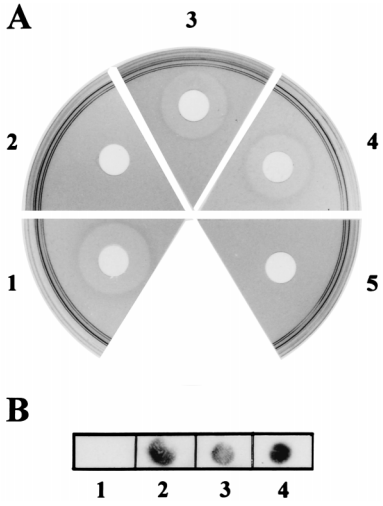

FIG. 3. (A) Lacticin 481 inhibitory effect on the following *L. lactis* strains: IL-kP, which does not contain any of the *lct* genes (panel 1); IL-FEG, expressing *lctF*, *lctE*, and *lctG* (panel 2); IL-EG, expressing *lctE* and *lctG* (panel 3); IL-FG, expressing *lctF* and *lctG* (panel 4); the wild-type lacticin 481 producer ADRIA 85LO30 (panel 5). Wells contain 100 μl of the wild-type *L. lactis* ADRIA 85LO30 filtered culture supernatant. (B) Dot blot analysis of total RNAs using as the probe the 0.9-kb *Sty*I-*Xmn*I DNA fragment (3′ end of *lctE* and complete *lctG*). RNAs were prepared from the following *L. lactis* strains: IL-kP (panel 1), IL-FEG (panel 2), IL-EG (panel 3), and IL-FG (panel 4).

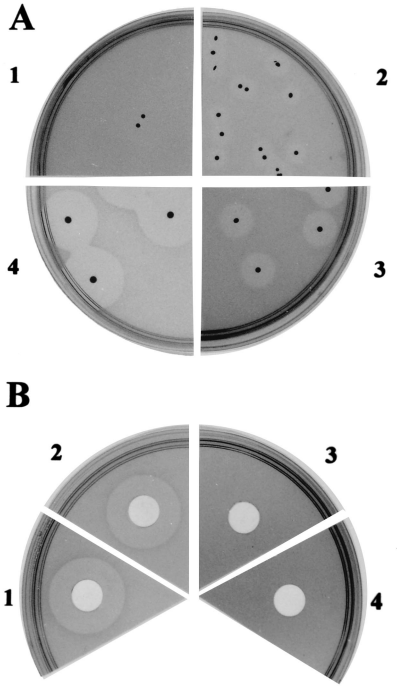

FIG. 4. Lacticin 481 production (A) and immunity (B) assays with the following *L. lactis* strains: IL1403, containing the pBS-pIL vector (panels 1); IL57, expressing *lctA* and *lctM* (panels 2); IL94, which contains the complete lacticin 481 operon (panels 3); the wild-type lacticin 481 producer ADRIA 85LO30 (panels 4). Lacticin 481 production was assayed by the solid agar medium method, whereas immunity was tested by the culture supernatant diffusion method.

We verified that an IL1835 supernatant concentrated to the same extent did not produce an inhibitory halo when tested against a lacticin 481-sensitive strain. One hundred microliters of dilutions of the concentrated bacteriocin was loaded into wells of an agar plate previously inoculated with the test strain, and the MIC was the lacticin 481 concentration which produced a 1-mm halo. *L. lactis* IL1403 containing either pEB-FEG (*lctF*, *lctE*, and *lctG* under the control of the strong constitutive promoter P32) or pEB118 (*lctA*, *lctF*, *lctE*, and *lctG*, whose expression is controlled by the wild-type promoters P1, P2, and P3 and the terminator T1, respectively [Fig. 1B]) showed higher immunity levels than ADRIA 85LO30 (Table 1). This can be explained by a higher level of expression of *lctF*, *lctE*, and *lctG* in the recombinant IL1403 strains than in ADRIA 85LO30. A high level of expression from pEB-FEG results probably from the use of the promoter P32 and from the high copy number of the vector pIL253, estimated as 45 to 85 copies per chromosome equivalent (39). As discussed above, pEB118 probably expresses *lctF*, *lctE*, and *lctG* at a lower level than pEB-FEG because of the terminator sequence T1. However, the expression might still be higher than that in ADRIA 85LO30 because pEB118 is also based on the high-copy-number vector pIL253, whereas the lacticin 481 operon in ADRIA 85LO30 is included in the 70-kb plasmid pOS5. The latter is likely to have a low copy number, due to its large size. To draw a better comparison with ADRIA 85LO30, we constructed pEB150, a low-copy-number variant of pEB118, by replacing pIL253 with pIL252. A copy number of six to nine per chromosome equivalent was reported for pIL252 (39). *L. lactis* IL1403 containing pEB150 showed an intermediate level of immunity: 8-fold higher than IL1403 harboring a fusion of pIL252 and pBluescript, 16-fold lower than ADRIA 85LO30, and 32-fold lower than IL1403 containing pEB118 (Table 1). The approximately 10-fold reduction in copy number between pEB118 and pEB150 resulted thus in a 32-fold reduction in the level of immunity.

The level of immunity obtained in *L. lactis* IL1403 by expressing *lctF*, *lctE*, and *lctG* is thus related to the level of expression of the genes, which could be influenced by (i) the promoters used, (ii) the regulation of these promoters, and (iii) the copy number of the plasmid carrying the genes. The promoters in pEB150 and the lacticin 481 operon are the same, and the copy numbers of pEB150 and pOS5 should be close. However, the activity of the lacticin 481 promoters might be positively regulated in *L. lactis* ADRIA 85LO30 but not in IL1403. The immunity conferred by *lctF*, *lctE*, and *lctG* in

TABLE 1. Levels of immunity to lacticin 481 of *L. lactis* ADRIA 85LO30 and recombinant IL1403 strains

L. lactis strain (plasmid)	Characteristic(s)	Plasmid copy no.[a]	Immunity level (MIC[b] [AU · ml^{-1}])
ADRIA 85LO30	Wild-type strain		640
IL1403(pBS-pIL253)	No *lct* gene	High	10
IL1403(pEB-FEG)	P32[c] *lctF lctE lctG*	High	>1,280[d]
IL1403(pEB118)	*lctA lctF lctE lctG*	High	1,280
IL1403(pEB94)	*lctA lctM lctT lctF lctE lctG*	High	1,280
IL1403(pBS-pIL252)	No *lct* gene	Low	5
IL1403(pEB150)	*lctA lctF lctE lctG*	Low	40
IL1403(pEB170)	*lctA lctM lctT lctF lctE lctG*	Low	60

[a] High and low copy numbers: 45 to 85 and 6 to 9 per equivalent chromosome, respectively.
[b] Lacticin 481 concentration producing a 1-mm halo against the test strain by the supernatant diffusion method. At least three determinations were made for each strain.
[c] The *lct* genes were expressed from P32, a strong constitutive promoter.
[d] Strain not inhibited by the highest lacticin 481 concentration used.

ADRIA 85LO30 would thus account for about 6% of the total immunity if the expression is the same as that from pEB150 and could account for up to 100% of the immunity if the activity of the promoters of the lacticin 481 operon is positively regulated. One hundred percent of the immunity would be achieved with an increase of transcription by less than 10, as indicated by the comparison between pEB118 and pEB150.

The levels of immunity conferred by the lacticin 481 operon cloned into pIL253 (pEB94) and pIL252 (pEB170) were close to the levels obtained with pEB118 and pEB150, respectively (Table 1). These results show that *lctM* and *lctT* are not primarily involved in immunity and have either no or only little effect on *lctF*, *lctE*, and *lctG* in the IL1403 background.

DISCUSSION

We previously reported *lctA*, *lctM*, and *lctT*, the first three genes of the lacticin 481 operon (31), *lctA* being the bacteriocin structural gene (27). A terminator-like sequence was observed between *lctA* and *lctM*, and Northern blotting with *lctA* probes detected transcripts containing only *lctA* (27, 31). However, Piard et al. could also detect with an *lctA* probe a 7- to 9-kb transcript of lower abundance (27). These data plus the fact that *lctM* was requisite for lacticin 481 biosynthesis (31) suggest that transcription could extend from *lctA* to *lctM*, *lctT*, and possibly an additional gene(s). The terminator sequence would thus act as an attenuator, ensuring a higher transcription level of *lctA* than of the other ORFs of the operon. Here, we complete the characterization of the operon by reporting three additional genes, *lctF*, *lctE*, and *lctG*, located downstream of *lctT* and proposed to be part of the *lctA* transcription unit. As a terminator-like sequence is found downstream of *lctG* and the gene upstream of *lctA* is very unlikely to be linked to the bacteriocin system, we propose that the lacticin 481 operon is composed of the six *lct* genes. The 7- to 9-kb transcript mentioned above would be of the appropriate length to include all six genes. This does not preclude the possibility that the lacticin 481 gene cluster could comprise an additional transcription unit(s) or that other unlinked genes of the producer strain could also play a role in lacticin 481 synthesis and/or immunity.

On the basis of the observed similarities, LctF, LctE, and LctG could be linked to form one ABC transporter. LctF would be the ATP binding domain, and LctE and LctG would be the two transmembrane domains. The transporter would thus be composed of two molecules of LctF associated with one LctE and one LctG molecule. The *lctE* start codon overlaps with the *lctF* stop codon, and the *lctG* start codon overlaps the last two codons of *lctE*. As suggested by van de Guchte et al. (44), such a structure could induce a translational coupling of the three genes, ensuring production of the three proteins in the appropriate ratio. Similar organizations of overlapping structure were found with the *nisF*, *nisE*, and *nisG* genes and the *epiF*, *epiE*, and *epiG* genes of the nisin and epidermin operons (25, 37). The facts that proteins having the highest degree of similarity with LctF, LctE, and LctG are involved in lantibiotic immunity and that coexpression of *lctF*, *lctE*, and *lctG* permitted *L. lactis* IL1403 to become insensitive to lacticin 481 let us conclude that the genes are involved in immunity to the bacteriocin. Deletion of any of these genes showed that all three genes are necessary to obtain the immunity phenotype. This strengthens the hypothesis that LctF, LctE, and LctG could be the subunits of one ABC transporter. The lacticin 481 operon is thus proposed to encode two ABC transporters, LctT and LctF/E/G, raising the question of the specificity of each transporter. Since the lacticin 481 operon with *lctM* and *lctT* deleted conferred a similar level of immunity as the complete operon, LctT does not seem to contribute to the immunity but is rather exclusively devoted to the cleavage and export of newly synthesized bacteriocin.

In comparison with the other gene clusters of the group A lantibiotics (38), the lacticin 481 system seems less complex, but additional genes might be part of the lacticin 481 gene cluster. As with the lantibiotics of the lactocin S subgroup, only one gene, *lctM*, seems to be responsible for the amino acid modifications and thioether ring formations. These modifications require two or three genes for bacteriocins of the nisin subgroup (for subtilin and nisin, *lanB* and *lanC* and, in addition, *lanD* in the case of epidermin). Only one protein, LctT, seems responsible for leader peptide cleavage and export of lacticin 481 while these two functions are dependent on two proteins, a protease, LanP, and a transporter, LanT, in most of the other lantibiotic systems (nisin, epidermin, Pep5, cytolysin, and lactocin S). An immunity system based on an ABC transporter seems to be common to lantibiotics, irrespective of their subgrouping, since it has already been found for nisin, subtilin, and epidermin (35, 38). This common immunity mechanism might be the consequence of a similar mode of action for the lantibiotics: the formation of pores in the cytoplasmic membrane of the target cells without the need for a membrane-associated receptor (1, 17, 32). In this case, an ABC transporter may provide immunity by active extrusion of bacteriocin molecules, keeping the lantibiotic concentration in the cytoplasmic membrane below the critical level which is necessary for the appearance of pores, as suggested by Peschel and Götz (25). However, at least two mechanisms of self-protection coexist in the nisin and subtilin cases, the second one relying on LanI, a lipoprotein anchored in the membrane and exposed on its extracellular surface. LanI would interact with the lantibiotic before the latter can reach the membrane (1). Although evaluations of the contribution of each mechanism give rise to conflicting data, it seems clear that they are each responsible for only a (sometimes small) part of the bacteriocin immunity (35). A *lanI* gene was also found in the Pep5 gene cluster, but no gene coding for an ABC transporter was reported (38). In contrast, no *lanI* gene was found in the epidermin gene cluster (38), but the ABC transporter EpiF/E/G seems insufficient to provide complete self-protection, suggesting the existence of other immunity factors (35). The level of immunity conferred by *lctF*, *lctE*, and *lctG* was dependent on the plasmid copy number, which probably influenced their level of expression. This made it difficult to estimate precisely their contribution to the wild-type level of lacticin 481 immunity. They could account for the totality of the immunity, but it would require a positive regulation of the lacticin 481 operon in the wild-type strain. Alternatively, we cannot exclude the possibility that a LanI counterpart or another immunity factor is needed to obtain full immunity. If this factor exists, it is encoded by a gene found in a transcription unit distinct from that of *lctA*. The lacticin 481 operon does not include regulatory genes such as the *lanR* and *lanK* genes of the nisin and subtilin gene clusters or the *epiQ* gene of the epidermin cluster (38). However, regulatory proteins could also be involved in the lacticin 481 system. In this case, their genes would be part of an additional transcription unit(s).

Our expression experiments showed, as previously reported (31), that introduction of only *lctA* and *lctM* in *L. lactis* IL1403 is sufficient to induce the production of active bacteriocin. This result now seems surprising, considering that both the cleavage-export and immunity systems are missing. The *L. lactis* IL1403 chromosome was shown to contain two genes, which are similar to and can complement *lcnC* and *lcnD* (46). LcnC and LcnD are responsible for the cleavage and secretion of

lactococcin A, a small nonlantibiotic bacteriocin (41). As LcnC is similar to LctT and to the other ABC transporters with an N-terminal protease domain (12) and as the cleavage sites of lacticin 481 and lactococcin A are similar (13), it is conceivable that the absence of *lctT* was complemented by the *lcnC* counterpart, allowing cleavage and export of lacticin 481. The situation regarding immunity was different since IL1403 containing *lctA* and *lctM* was still sensitive to lacticin 481: no chromosomal IL1403 gene could therefore complement the absence of the immunity genes. Although the bacteria could survive and grow, they showed an impaired growth rate (generation time, 65 min versus 35 min for IL1403 without plasmid) and their inhibitory activity was quite low compared to that of the wild-type lacticin 481 producer. This might be a consequence of the slower growth of the strain and/or of a nonoptimal complementation of *lctT*. The concentration of accumulated lacticin 481 was close to the MIC needed to inhibit IL1403 (about 10 $AU \cdot ml^{-1}$), which explains the survival of the producer bacteria. IL1403 containing the complete lacticin 481 operon in a high-copy-number plasmid had a normal growth rate (generation time, 36 min) and stronger inhibitory activity. This activity was, however, still reduced compared to that of the wild type. This reduction could be due to a competition between the IL1403 LcnC and LctT. Also, we cannot exclude the possibility that an additional gene(s) other than the six *lct* genes is (are) involved in lacticin 481 biology. If such a gene exists, either it is largely dispensable for lacticin 481 biosynthesis and immunity or *L. lactis* IL1403 provides a counterpart.

From the application point of view, our results show that we are able to obtain the lacticin 481 immunity phenotype in an *L. lactis* strain by coexpressing *lctF*, *lctE*, and *lctG*. Consequently, we may be able to construct new starter strains, resistant to lacticin 481, a prerequisite condition for its use in an industrial process. Furthermore, the reconstruction of the entire operon shows that we should be able to construct starter strains producing lacticin 481.

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