

# Genes Involved in Immunity to and Secretion of Aureocin A53, an Atypical Class II Bacteriocin Produced by *Staphylococcus aureus* A53

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**Aureocin A53 is an antimicrobial peptide produced by *Staphylococcus aureus* A53. The genetic determinants involved in aureocin A53 production and immunity to its action are organized in at least four transcriptional units encoded by the 10.4-kb plasmid pRJ9. One transcriptional unit carries only the bacteriocin structural gene, *aucA*. No immunity gene is found downstream of *aucA*, as part of the same transcriptional unit. Further downstream of *aucA* is found an operon which contains the three genes *aucEFG*, whose products seem to associate to form a dedicated ABC transporter. When *aucEFG* were expressed in RN4220, an aureocin A53-sensitive *S. aureus* strain, this strain became partially resistant to the bacteriocin. A gene disruption mutant in *aucE* was defective in aureocin A53 externalization and more sensitive to aureocin A53 than the wild-type strain, showing that *aucEFG* are involved in immunity to aureocin A53 by active extrusion of the bacteriocin. Full resistance to aureocin A53 was exhibited by transformants carrying, besides *aucEFG*, the operon formed by two genes, *aucIB* and *aucIA*, located between *aucA* and *aucEFG* and carried in the opposite strand. *AucIA* and *AucIB* share similarities with hypothetical proteins not found in the gene clusters of other bacteriocins. A gene disruption mutant in *orf8*, located upstream of *aucA* and whose product exhibits about 50% similarity to a number of hypothetical membrane proteins found in many Gram-positive bacteria, was strongly affected in aureocin A53 externalization but resistant to aureocin A53, suggesting that Orf8 is also involved in aureocin A53 secretion.**

**B**acteriocins (Bac) are a group of antimicrobial peptides which are produced by bacteria inhabiting diverse environments. They typically inhibit the growth of strains closely related to the producer strain although some bacteriocins have a broad spectrum of activity inhibiting different species of bacteria (21). Bacteriocins produced by Gram-positive bacteria are currently classified into three major classes although a fourth class, composed of cyclic bacteriocins, has already been proposed (40). Class I bacteriocins, the lantibiotics, are small, heat-stable peptides that contain modified amino acids and undergo extensive posttranslational modifications (6). Class II bacteriocins are small, heat-stable peptides which do not contain modified amino acids (29). Class III bacteriocins are heat-labile antimicrobial proteins (5, 21). The majority of the peptide bacteriocins act by permeabilization of the membrane of the sensitive cells, leading to leakage of cellular solutes and, eventually, to cell death (21).

Bacteriocin production is invariably linked to the expression of specific immunity systems which are required for producer self-protection against the toxicity of the bacteriocin (6, 21, 29). For lantibiotics, the protection is mediated by the so-called immunity proteins (LanI or LanH) and/or dedicated ABC transporter proteins (LanEFG). For instance, immunity to the lantibiotic nisin involves a combined action which includes (i) sequestering of the bacteriocin molecules on the bacterial cell membrane by NisI (15) and (ii) removal of the bacteriocin from cells by NisFEG (35). LanH is also a lantibiotic-binding protein which captures the bacteriocin in an energy-independent manner, after which the lantibiotic is transported to the extracellular space by LanEFG in an energy-dependent manner (31). Although LanH presents a function similar to that of LanI (lantibiotic binding), the structures of the two proteins are quite different. For example, NukH, a protein involved in immunity to nukacin ISK-1, is a membrane protein

with three transmembrane domains, and its N terminus is located on the cytoplasmic side (31). On the other hand, NisI is a lipoprotein anchored to the extracellular side of the membrane by its N-terminal lipid (39).

In class II bacteriocins, immunity proteins are generally also involved in self-protection. The genetic determinants proposed or confirmed to confer immunity are frequently found closely associated with the bacteriocin structural gene(s), generally as part of the same operon (29). Their products usually have a high pI, are small (ranging from 51 to approximately 150 amino acids), and contain either no or up to four putative transmembrane segments (26). This fact suggests that the immunity proteins can be secreted out of the cell or even be retained in the membrane (14, 16). It has been shown that, for class II bacteriocins that use components of the mannose phosphotransferase system of susceptible cells as targets/receptors, the immunity protein forms a strong complex with the receptor proteins and the bacteriocin and thereby prevents cells from being killed (13).

Another mechanism of immunity has been described for some class II bacteriocins, which relies on the activity of a multidrug transporter protein (19). It seems that these multidrug transport-

Received 16 September 2011 Accepted 1 December 2011

Published ahead of print 9 December 2011

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doi:10.1128/JB.06203-11

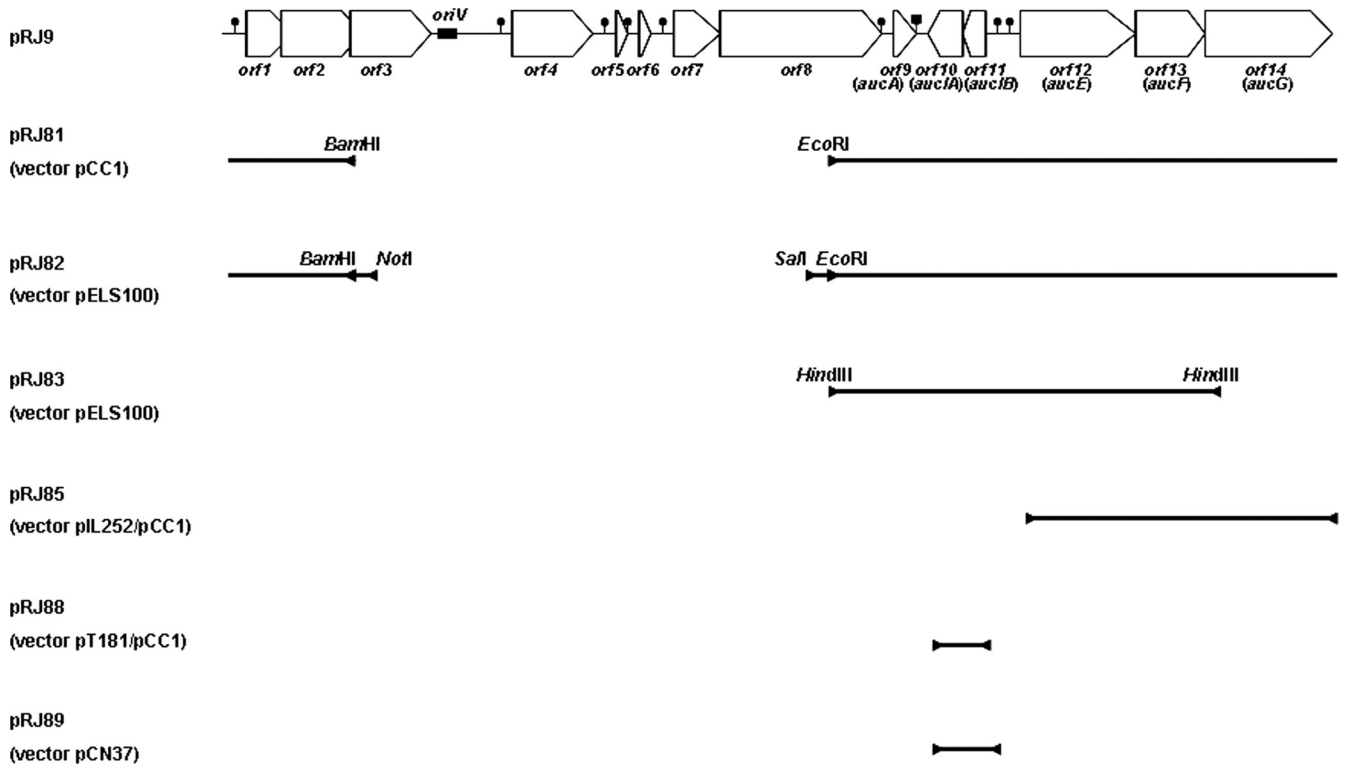


FIG 1 Genetic map of plasmid pRJ9 (adapted from Fig. 1 of reference 27 with permission of the publisher) showing the inserts cloned into different vectors. The main restriction sites used in the cloning experiments are shown. The inserts found in pRJ85, pRJ88, and pRJ89 were obtained by PCR amplification of the corresponding regions.

ers mediate bacteriocin immunity by removing bacteriocin that enters the cytoplasmic membrane from the outside. Besides conferring immunity, these transporters also function as exit pumps for the bacteriocins, extruding the molecules from their site of production, the cytoplasm, to the extracellular medium (19).

Aureocin A53 is an atypical class II bacteriocin produced by *Staphylococcus aureus* A53 and encoded by a 10.4-kb plasmid named pRJ9 (20). This bacteriocin is highly active against multidrug-resistant nosocomial staphylococcal strains, including the multidrug-resistant *S. aureus* (MRSA) clone endemic in most Brazilian hospitals and in other countries (25), *Streptococcus agalactiae*, and coagulase-positive staphylococcal strains involved in bovine mastitis, a disease of great economic impact (9, 12). It can also inhibit *Listeria monocytogenes*, an important food-borne pathogen (11). Therefore, aureocin A53 has potential in medical applications and also as a food preservative. However, optimal and rational exploitation of aureocin A53 as an antimicrobial agent requires its production in large scale. This, in turn, necessitates knowledge on all gene products required for its production.

The complete sequence of pRJ9 showed that this plasmid presents 14 putative open reading frames (ORFs) (Fig. 1) (27). One segment of pRJ9 (*orf1* to *orf6*) contains functions related to plasmid mobilization and replication. The second part (*orf7* to *orf14*) appears to carry genes involved in the production of aureocin A53. No gene encoding an immunity protein has been identified in the transcriptional unity which carries the aureocin A53 structural gene, *aucA*. However, three genes (*orf12*, *orf13*, and *orf14*) were identified in pRJ9, whose products possess similarity to three-component ABC transporters generally involved in immunity to

either lantibiotics (6) or cyclic bacteriocins (29, 40). In the present work, the role of these genes and of a putative operon formed by *orf11* and *orf10*, found upstream of *orf12*, *orf13*, and *orf14*, in immunity to aureocin A53 was investigated. During the course of this study another gene, *orf8*, involved in aureocin A53 secretion was also detected.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains used in the present study are listed in Table 1. *Escherichia coli* strains were cultivated at 37°C in Luria-Bertani (LB) broth supplemented with 100  $\mu\text{g ml}^{-1}$  ampicillin (Ap), 150  $\mu\text{g ml}^{-1}$  erythromycin (Em), or 12.5  $\mu\text{g ml}^{-1}$  chloramphenicol (Cm), when appropriate. *Lactobacillus* species strains were grown in Man-Rogosa-Sharpe (MRS; Oxoid) broth at 30°C. When appropriate, the MRS medium was supplemented with Em at 5  $\mu\text{g ml}^{-1}$ . *S. aureus* and *Micrococcus luteus* were grown at 37°C in brain heart infusion (BHI) broth (Difco). *S. aureus* strains carrying pRJ9 derivatives upon transposon mutagenesis were grown in the presence of 10  $\mu\text{g ml}^{-1}$  Em. *S. aureus* strains carrying recombinant plasmids derived from the cloning experiments were grown in the presence of either Em (for derivatives of pCN37 or pIL252) or tetracycline ([Tc] for derivatives of pT181), both at 10  $\mu\text{g ml}^{-1}$ . All media were supplemented with agar at 1.5% or 0.6% (wt/vol), when required.

**In silico sequence analyses.** Sequence similarity of the predicted amino acid sequences of the proteins encoded by pRJ9 to other proteins was searched using the Basic Local Alignment Search Tool (BLASTn) analysis. Multiple sequence alignments were carried out using BioEdit software. Protein transmembrane domains were predicted by using the TMpred Server ([http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)). The pRJ9 DNA sequence was retrieved from GenBank (accession number AF4478813).

TABLE 1 Plasmids and bacterial strains used in this work

Plasmid or strain	Relevant feature(s) <sup>a</sup>	Reference or source
<b>Plasmids</b>		
pCC1	8.1 kb; Cm <sup>r</sup> <i>ori2 oriV</i>	Epicentre
pCR2.1-TOPO	3.9 kb; Km <sup>r</sup>	Invitrogen
pELS100	7.0 kb; shuttle vector <i>E. coli</i> - <i>Lactobacillus</i> ; Ap <sup>r</sup> Em <sup>r</sup>	LMGT
pIL252	4.6 kb; a LAB vector; Em <sup>r</sup>	36
pNO6	12.7 kb; shuttle vector <i>E. coli</i> /LAB formed by ligation of pCC1 and pIL252; Cm <sup>r</sup> Em <sup>r</sup>	This work
pCN37	6.3 kb; shuttle vector <i>E. coli</i> - <i>S. aureus</i> ; Ap <sup>r</sup> Em <sup>r</sup>	8
pT181 <i>mcs</i>	4.8 kb; <i>S. aureus</i> plasmid; Tc <sup>r</sup>	2
pNO9	12.9 kb; shuttle vector <i>E. coli</i> / <i>S. aureus</i> formed by ligation of pCC1 and pT181 <i>mcs</i> ; Cm <sup>r</sup> Tc <sup>r</sup>	
pRJ9	10.4 kb; codes for aureocin A53	20
pRJ81	13.9 kb; Cm <sup>r</sup> ; BamHI/EcoRI-A fragment of pRJ9 cloned into pCC1	This work
pRJ82	13.1 kb; BamHI/EcoRI-A fragment of pRJ9 cloned into pELS100	This work
pRJ83	10.6 kb; HindIII-A fragment of pRJ9 cloned into pELS100	This work
pRJ84	11.0 kb; <i>orf12-orf13-orf14</i> of pRJ9 cloned into pCC1	This work
pRJ85	15.6 kb; <i>orf12-orf13-orf14</i> cloned into pNO6	This work
pRJ88	13.2 kb; <i>orf11-orf10</i> cloned into pCC1 and ligated to pT181	This work
pRJ89	7.0 kb; <i>orf11-orf10</i> cloned into pCN37	This work
<b>Strains</b>		
<i>E. coli</i> strains		
EPI300		Epicentre
DH5 $\alpha$	<i>lacZ</i> $\Delta$ M15 <i>hsdR</i> mutant	33
<i>L. sakei</i> DSM 20017	Sensitive to aureocin A53	LMGT
<i>M. luteus</i> ATCC 4698	Sensitive to aureocin A53	ATCC
<i>S. aureus</i> strains		
RN4220	<i>hsd</i> mutant; sensitive to aureocin A53	30
RN9590	RN4220 carrying pCN37	8
A53	pRJ9; 10.4 kb; Bac <sup>+</sup> Imm <sup>+</sup>	20
MB38	pRJ14 (pRJ9::Tn551); 15.6 kb; Em <sup>r</sup>	1
MB139	pRJ38 (pRJ9::Tn917- <i>lac</i> ); 18.9 kb; Em <sup>r</sup>	11
MB140	pRJ39 (pRJ9::Tn917- <i>lac</i> ); 18.9 kb; Em <sup>r</sup>	11
MB143	pRJ40 (pRJ9::Tn917- <i>lac</i> ); 18.9 kb; Em <sup>r</sup>	11
MB145	pRJ108 (pRJ9::Tn917- <i>lac</i> ); 18.9 kb; Em <sup>r</sup> ; low aureocin A53 production	This work
MB388	RN4220 carrying pT181 <i>mcs</i>	This work
MB420	RN4220 carrying pRJ88	This work
MB422	RN4220 carrying pRJ85 and pRJ88	This work
MB425	RN4220 carrying pNO6	This work
MB426	RN4220 carrying pRJ85	This work
MB439	RN4220 carrying pRJ89	This work
MB502	Plasmidless derivative of strain MB145	This work

<sup>a</sup> Imm<sup>+</sup>, presence of immunity to aureocin A53; *hsd* mutant, deficient in DNA restriction.

**DNA techniques.** Plasmids pCC1, pELS100, pIL252, pNO6, pNO9, and pCN37 and their derivatives carrying inserts were isolated from the *E. coli* host strains by using a QiaPrep Spin Miniprep kit (Qiagen) according to the supplier's instructions. The same kit was used to purify plasmid DNA from either lactic acid bacteria (LAB) or *S. aureus*. In both cases, the extractions were performed after treatment of the cells, for 30 min at 37°C, with a lysis solution (0 mM Tris-HCl, pH 8.0, 40 mM EDTA, and 25% [wt/vol] sucrose) supplemented with either 5 mg ml<sup>-1</sup> lysozyme (Sigma), 15 U ml<sup>-1</sup> mutanolysin (Sigma), 100  $\mu$ g ml<sup>-1</sup> RNase A (Sigma), and 2.5  $\mu$ g ml<sup>-1</sup> SDS, for LAB cells, or 1 mg ml<sup>-1</sup> lysozyme, 20  $\mu$ g ml<sup>-1</sup> lyso-staphin (Sigma), 100  $\mu$ g ml<sup>-1</sup> RNase A, and 2.5  $\mu$ g ml<sup>-1</sup> SDS, for the staphylococcal cells. Restriction enzymes were purchased from Promega, and T4 DNA-ligase was from Invitrogen. The enzymes were used as specified by the suppliers. Agarose gel electrophoreses were performed by standard methods (33).

PCR amplification of the fragment containing the putative *orf12-orf13-orf14* operon of pRJ9 was performed using the primers J010A and J011 (Table 2). Each reaction mixture contained the following: 1 $\times$  Pfx reaction mix, 1.0 mM MgSO<sub>4</sub>, 10  $\mu$ M each primer, 1 U of platinum Pfx

DNA polymerase (Invitrogen), and 200 ng of template DNA. PCR amplification of the fragment containing the putative *orf11-orf10* operon of pRJ9 was performed using the primers ORF10F and ORF11R (Table 2) and Phusion High-Fidelity DNA-Polymerase (Finnzymes Oy). The amplification cycles were performed following the suppliers' recommendations. PCR fragments for cloning experiments were purified by a Qiaex II kit (Qiagen). The nucleotide sequence of the amplicons was verified by dideoxy chain termination sequencing in an automated sequencer (ABI Prism 3100; Perkin Elmer) after the amplicons were cloned into the plasmid vector pCR2.1-TOPO (Invitrogen), according to the manufacturer's instructions.

**Plasmid constructions.** All plasmids constructed in this study are also described in Table 1 and shown in Fig. 1. To generate plasmid pRJ81, pCC1 and pRJ9 were cleaved with BamHI and EcoRI. Digestion of pRJ9 generated two fragments of 5.8 kb and 4.6 kb. The 5.8-kb fragment, encompassing the region from the 3' end of *orf8* to the first two thirds of *orf2* (Fig. 1), was ligated to linearized pCC1. The resulting plasmid, called pRJ81, was introduced into chemically competent *E. coli* EPI300.

For construction of pRJ82, plasmids pRJ81 and pELS100, a shuttle

TABLE 2 Primers used in the present study

Primer	Sequence (5' → 3')	$T_m$ (°C) <sup>a</sup>	Expected amplicon size (bp)
J010A	GGA TCC AAT AAA CAT CAC CCT TAT GT	55	3,041
J011	GGT GGT GTCGAC GGG ATA TTC CCC AAT CCC CTT	68	
ORF12-13F	AGA GTG AAG TTA GAG ATG GG	51	381
ORF12-13R	TTT CCT GAC CCA GAA GGC	55	
ORF13-14F	TAT CCG GTG GCC AAC AAC	55	366
ORF13-14R	GTA ATT ACT GAA GAG ATG CCC	51	
ORF10F	GTT GAA TAA AGA GCC AAA GGT GCC A	56	735
ORF11R	CAA TTT TAT TCC TCC ATT TCA GAT GA	52	
ORF10-11F	TTG GAT GCT TGG TTA GAA AA	50	576
ORF10-11R	TTA TCC TTG ATA ATC TTT AGC	48	
GyrF	GCG TGA ATC ATT TTT AGA TTA TGC G	53	447
GyrR	AAG TTA GGG AAT CGA GCA G	48	

<sup>a</sup> Thermal denaturation midpoint temperature.

vector for *E. coli* and *Lactobacillus* spp., were digested with Sall and NotI. The digestion of pRJ81 generated five fragments, and the 6.15-kb fragment (Fig. 1) was isolated and ligated into the linearized pELS100. The new plasmid, called pRJ82, was transferred first to the chemically competent *E. coli* EPI300 strain and, subsequently, to *Lactobacillus sakei* DSM20017 by electroporation.

For construction of pRJ83, plasmids pELS100 and pRJ9 were digested with HindIII. These digestions linearized plasmid pELS100 and resulted in four fragments of plasmid pRJ9. The largest one, fragment HindIII-A (3,581 bp; from the 3' end of *orf8* to the 5' end of *orf14*) (Fig. 1), was gel purified and ligated to pELS100. pRJ83 was transferred to *E. coli* EPI300 and, subsequently, to *L. sakei* DSM20017.

To construct pRJ85, a fragment containing *orf12*, *orf13*, and *orf14* was amplified by PCR. This amplicon was first ligated to plasmid pCR2.1-TOPO and transferred to chemically competent *E. coli* DH5 $\alpha$ . Five kanamycin-resistant transformants were chosen for plasmid DNA extraction and sequencing to confirm the correct amplicon sequence prior to digestion with EcoRI to liberate the insert. The insert was ligated to pCC1, also digested with EcoRI, generating plasmid pRJ84. Subsequently, pRJ84 was ligated to pIL252, a LAB vector, by the unique BamHI site present on both plasmids, generating plasmid pRJ85. pRJ85 was transferred to *E. coli* EPI300 and, subsequently, to *L. sakei* DSM20017.

Plasmid pRJ88, containing an amplicon carrying both *orf11* and *orf10*, was constructed in a manner similar to that described for plasmid pRJ85 but using pT181mcs, a staphylococcal plasmid, instead of pIL252, as a Gram-positive vector. pRJ89 was generated by cloning the amplicon carrying both *orf11* and *orf10* into the unique EcoRI site of pCN37, an *E. coli*/S. aureus shuttle vector. Both plasmids were transferred to *E. coli* DH5 $\alpha$  and, subsequently, to *S. aureus* RN4220 by transformations.

The presence of the correct fragment in all recombinant plasmids was verified by agarose gel electrophoresis, restriction endonuclease analyses, PCR amplification, and DNA sequencing, when appropriate.

**Transformations.** Transformation of chemically competent *E. coli* cells was performed by standard methods (33). LAB and staphylococci were transformed by electroporation according to Aukrust et al. (3) and Schenk and Laddaga (34), respectively.

**Plasmid-curing experiments.** Strain MB145, the host strain of plasmid pRJ108, was used for curing experiments. pRJ108 is a pRJ9-derivative tagged with Tn917-*lac*, which was obtained by the method described by Oliveira et al. (11). MB145 cells were incubated in 5 ml of tryptic soy broth (TSB) medium (Difco) for 24 h at 45°C, after which the culture was diluted 1,000-fold in the same medium and reincubated under the same conditions. This step was repeated until the 10th day, when the culture was serially diluted in 0.85% saline, and dilutions of 10<sup>-6</sup> and 10<sup>-7</sup> were plated onto TSB agar plates. After incubation at 37°C for 18 h, the colonies were replica plated into Em (10  $\mu$ g ml<sup>-1</sup>)-containing medium to test for loss of the erythromycin resistance. Plasmid DNA was isolated from Em<sup>s</sup>

colonies and subjected to electrophoresis in agarose gel to confirm plasmid loss. The sensitivity of the cured derivatives to aureocin A53 was tested by a deferred-antagonism assay on solid BHI medium, as described by Giambiagi-deMarval et al. (20), using them as the indicator and the wild-type strain, A53, as the bacteriocin producer.

**Analysis of the toxicity of the inserts cloned into pCC1.** The toxicity of the insert present on pRJ81 was tested in *E. coli* by induction of the plasmid to a high copy number per cell, according to instructions of the Copy Control PCR cloning kit (Epicentre). Strains EPI300 and EPI300/pCC1 were also included in these experiments as controls. The strains were grown in 5 ml of LB medium at 37°C for 18 h and diluted to an optical density at 600 nm (OD<sub>600</sub>) of 0.05. Four hundred microliters from each culture was distributed in a Bioscreen honeycomb 100-well plate with addition of the copy control induction agent (Epicentre), according to the manufacturer's instructions. The diluted cultures without addition of the induction agent were used as controls. The growth curves were monitored at 37°C in a Bioscreen C system (Oy Growth Curves AB, Ltd.), which measured kinetically the culture OD<sub>600</sub> at time intervals of 30 min, for 24 h. Five replicas of the experiment were performed for each strain. Graphs originated from these measurements were generated using the program Growth Curves, version 2.28, for Microbiology Reader Bioscreen (Labsystems).

**Aureocin A53 preparations.** *S. aureus* A53 was grown in either 100 ml or 1 liter of BHI broth at 37°C, with vigorous shaking for 24 h. The cells were removed by centrifugation at 12,000  $\times$  g for 15 min at 4°C, and the supernatant was sterilized by membrane filtration (0.22- $\mu$ m pores; Sarstedt). Alternatively, aureocin A53 present in the culture supernatant was concentrated by ammonium sulfate precipitation (40%; wt/vol), re-suspended in either 10 ml or 100 ml of sterile Milli-Q water, and dialyzed against 0.1 M Na phosphate buffer, pH 6.5.

Antimicrobial activity in all preparations was determined by the dilution method using a microtiter plate assay as described by Ceotto and coworkers (7). Briefly, serial 2-fold dilutions of the bacteriocin preparation were prepared in 100  $\mu$ l of BHI broth. Each well was seeded with 100  $\mu$ l of the sensitive strain (10<sup>6</sup> cells of *M. luteus* ATCC 4698) in BHI broth. The plates were incubated for 18 h at 37°C. The bacteriocin activity was measured in bacteriocin units (BU) per ml, which represented the reciprocal of the highest supernatant dilution showing at least a 50% inhibition of the bacterial growth in relation to the control with no bacteriocin added, multiplied by 10.

**Determination of the aureocin A53 activity kinetics.** An experiment to determine aureocin A53 activity kinetics was performed by reading the OD<sub>600</sub> (in a Bioscreen C system) of an aureocin-susceptible culture in the presence of the partially purified bacteriocin at 37°C in intervals of 30 min for a period of 4 h. The partially purified aureocin A53 (640 BU), obtained by ammonium sulfate precipitation, was tested against 10<sup>9</sup> cells of the indicator strain, *M. luteus* ATCC 4698, in a 400- $\mu$ l final volume.



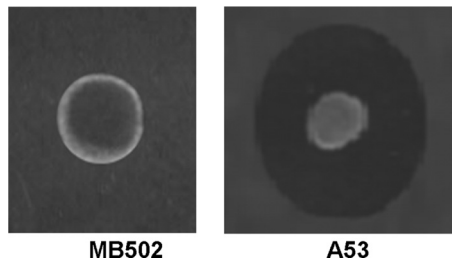


FIG 2 Sensitivity of strain *S. aureus* MB502 (a cured derivative of strain MB145 which lost plasmid pRJ108) to aureocin A53. Strains A53 and MB502 were used as bacteriocin producers, and strain MB502 was used as the indicator of aureocin A53 production.

**Immunity assays.** The immunity expression to aureocin A53 exhibited by the strains carrying either pRJ9 genes or pRJ9 derivatives tagged with a transposon was tested in microtiter plate assays containing the appropriate broth medium with 2-fold serial dilutions of an aureocin A53 preparation. The bacterial cells under test were collected at the stationary phase of growth, and the OD<sub>600</sub> was adjusted to 0.05 by the addition of the appropriate medium. In all assays, 100- $\mu$ l volumes of these suspensions were added to each well. Controls grown in the absence of the aureocin A53 were also included. The microtiter plates were incubated at the appropriate temperature, according to the bacteria tested, for 24 h, after which the OD<sub>620s</sub> were measured.

When needed, the immunity to aureocin A53 exhibited by randomly selected transformants was also determined using the Bioscreen C honeycomb 100-well plates as described for the toxicity assays of the inserts cloned into pCC1, with minor modifications. Briefly, 200  $\mu$ l of 2-fold serial dilutions of an aureocin A53 preparation was distributed in wells of a plate, after which 200  $\mu$ l from each culture, diluted to an OD<sub>600</sub> of 0.05 in the appropriate broth, was added to each well. The growth curves were monitored at either 30°C or 37°C at time intervals of 1 h for a period of 16 to 18 h. In all cases, the assays were performed at least in triplicate. Strains carrying only the vectors used in the cloning experiments, but without inserts, were used as controls.

**pRJ9 mutant studies.** Mutants of plasmid pRJ9 were obtained previously by transposon mutagenesis (11). The exact position of the insertion of the transposon into plasmid pRJ9 was determined in the present study by DNA sequencing, using an 18-bp primer (5'-TGT ACC ACT AAT AAC TCA-3') whose 3' end is located 101 bp upstream of the left end of the transposon. The presence of aureocin A53 in the culture supernatant and the immunity to aureocin A53 expressed by these mutants were analyzed as described above.

**Transcription analyses.** Isolation of total RNA from *S. aureus* strain A53 was performed with an RNeasy Mini kit (Qiagen), essentially as described by Coelho et al. (10). Briefly, cultures were harvested after 18 h of growth at 37°C. The cells were lysed by incubation with lysozyme (40 mg ml<sup>-1</sup>; Sigma) and lysostaphin (10 U; Sigma) in Tris-HCl-EDTA (TE) buffer. After this step, the procedure followed the manufacturer's recommendations. RNA preparations were quantified by using a NanoDrop ND1000 Spectrophotometer (Thermo Scientific). DNase I-pretreated RNA (500 ng) was subjected to cDNA synthesis using a Revert Aid H Minus First Strand cDNA Synthesis kit (Fermentas) as described by the manufacturer. Reverse transcription-PCRs (RT-PCRs) were performed with the following primers (Table 2): ORF12-13F and ORF12-13R for cotranscription analysis of *aucE* and *aucF*; ORF13-14F and ORF13-14R for cotranscription analysis of *aucF* and *aucG*; ORF10-11F and ORF10-11R for cotranscription analysis involving *orf11-orf10*. An internal control was performed using the primers GyrF and GyrR targeting the housekeeping gene *gyrA* that codes for the DNA-gyrase subunit A. In addition, a DNA contamination control was performed using this same pair of primers. This control was a PCR using the same conditions of the RT-PCR process without the RT step, and it demonstrated the purity of the RNA

preparation. The PCR mixtures consisted of 1 $\times$  reaction buffer, 1 U of DyNAzyme II DNA-polymerase (Finnzymes Oy), 2.5 mM MgCl<sub>2</sub>, 10 mM concentration of each deoxynucleoside triphosphate (dNTP; Promega), and 20 pmol of each primer. The PCRs were done essentially as described by Coelho et al. (10), and the amplicons were analyzed on 1% (wt/vol) agarose gels, using 100-bp DNA ladders (from either Fermentas or Promega) as size markers.

## RESULTS AND DISCUSSION

**Aureocin A53 activity kinetics.** In previous attempts, in spite of the many curing agents tested, we were unable to cure plasmid pRJ9 from strain A53 and, therefore, to prove that pRJ9 carries genetic determinants involved in aureocin A53 immunity (20). In order to investigate the reasons for this failure, the kinetics of aureocin A53 activity was tested against *M. luteus* in a Bioscreen C system. The OD<sub>600</sub> of the susceptible micrococcal culture was drastically reduced by the peptide action: from 1.8 to 1.4 after 15 min, to  $\sim$ 0.9 after 90 min (50% of reduction), and to  $\sim$ 0.6 after 4 h, showing that aureocin A53 has lytic activity (data not shown). The lytic activity of aureocin A53 may explain why derivatives of strain A53 devoid of plasmid pRJ9 could never be recovered previously. If pRJ9 carries genes involved in bacteriocin immunity, the plasmid loss is expected to result in cured derivatives which become sensitive to aureocin A53 produced by the remaining Bac<sup>+</sup> cells. Therefore, to avoid the interference of aureocin A53 in the curing experiments, strain MB145, carrying a pRJ9 mutant defective in bacteriocin production, was created by transposon mutagenesis.

**Curing of a plasmid pRJ9 derivative.** Strain MB145, carrying a pRJ9 derivative tagged with a transposon encoding Em resistance and producing a low level of bacteriocin (40 BU ml<sup>-1</sup>), was submitted to curing experiments. After 10 days of growth at 45°C, two colonies proved to be Em<sup>s</sup>. The plasmid loss was confirmed by agarose gel electrophoresis (data not shown). The sensitivity of both Em<sup>s</sup> colonies to aureocin A53 was also confirmed (Fig. 2; Table 3), indicating that plasmid pRJ9, indeed, carries at least one gene involved in bacteriocin immunity, as found in most bacterial systems (6, 29, 40).

**In silico analyses of the products encoded by *orf12*, *orf13*, and *orf14*.** The products of *orf12*, *orf13*, and *orf14* have 331, 226, and

TABLE 3 Production of aureocin A53 and immunity to aureocin A53 exhibited by strains either carrying mutations in pRJ9 or cured of pRJ9

Strain	Position of the transposon insertion (nt) <sup>a</sup>	Production of aureocin A53 (%) relative to that of strain A53 <sup>b</sup>	Smallest amt (BU) of aureocin A53 with inhibitory activity against the strain <sup>c</sup>
MB139(pRJ38)	<i>orf6</i> (4094–4095)	68	1,280
MB38(pRJ14)	<i>orf8</i> (5131–5132)	2.5	1,280
MB143(pRJ40)	<i>orf8</i> (5302–5303)	0.9	1,280
MB140(pRJ39)	<i>aucE</i> (7720–7721)	24	80
A53(pRJ9)	Wild-type plasmid	100	1,280
MB502	Cured of pRJ9	0	40

<sup>a</sup> Determined by DNA sequencing. The numbers in parentheses refer to nucleotide (nt) positions between which the transposon is inserted in each mutant.

<sup>b</sup> Production of aureocin A53 after growth in BHI medium at 37°C for 24 h. The bacteriocin secreted by strain A53 (3,140 BU ml<sup>-1</sup>) was set as 100%. Data are the means of three independent experiments.

<sup>c</sup> The susceptibility of each strain to aureocin A53 was tested in microtiter plate assays. Data are the means of three independent experiments.

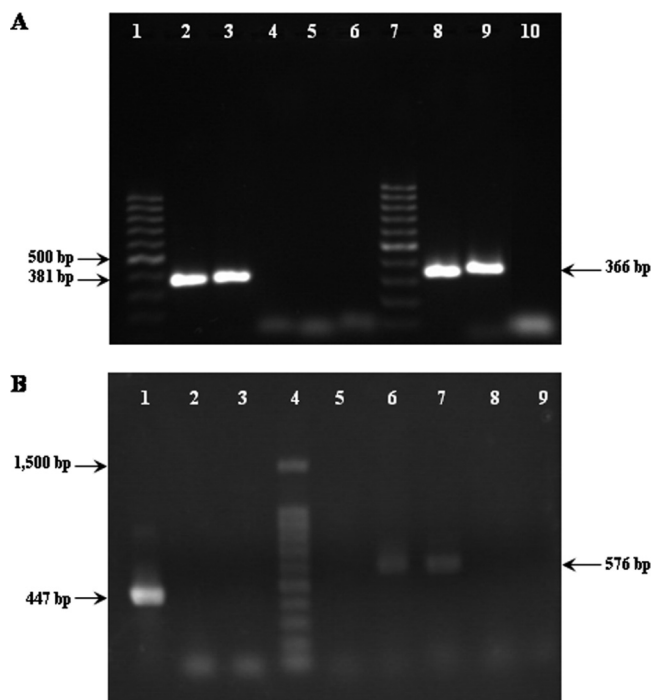
388 amino acids, respectively. The hydrophobicity profiles of these proteins were determined, and Orf12 and Orf14 were found to have apparently one and four transmembrane domains, respectively (data not shown), suggesting their insertion into the cytoplasmic membrane. On the other hand, Orf13, which does not possess transmembrane domains, shares domains with the consensus sequences of the ABC ATPases found in bacteria and involved in the transport of several bacteriocins (data not shown). These ATP-binding domains, which are responsible for ligation and hydrolysis of ATP, possess a conserved primary sequence whose main motifs, Walker A and Walker B (22, 28), are found in Orf13. Other sequences such as the switch region (which contains a histidine loop), motif D (which contains an invariant Asp), and the signature motif (LSGGQ) are also present in Orf13 (data not shown). Since *orf12*, *orf13*, and *orf14* products possess similarity to three-component ABC transporters found in some bacteriocin gene clusters and involved in immunity, they will be referred to here as *aucE*, *aucF*, and *aucG*, respectively.

**The *aucE*, *aucF*, and *aucG* genes of pRJ9 are transcribed as an operon.** Putative ribosome binding sites (RBS) were found upstream of each *auc* gene although a canonical one (AGGAGG) was not found preceding any gene. *aucE* and *aucG* start with a TTG codon, while *aucF* starts with an ATG codon (data not shown).

A putative SigA-dependent promoter is located upstream of *aucE*. A  $-10$  region, with five matches to the consensus sequence (TATAAT), and a  $-35$  region, with four matches to the consensus sequence (TTGACA) (the most conserved bases are shown in boldface), were found 46 bp and 69 bp, respectively, upstream of the start codon TTG, with 17 bp between them. RT-PCR analysis (Fig. 3A) detected amplicons corresponding to cotranscription of either *aucE* and *aucF* (lane 3) or *aucF* and *aucG* (lane 9), confirming that these genes are coordinately transcribed.

Northern blotting experiments detected in strains A53, MB38, and MB143 (the latter two strains carry a transposon insertion into *orf8* of pRJ9) a weak hybridization signal, corresponding to an mRNA of  $\sim 2.8$  kb (data not shown), a size similar to that expected for a transcript comprising the genes *aucEFG*. This result confirms that these genes are transcribed as an operon. The  $\sim 2.8$ -kb signal was not found in strain MB140 because, in this strain, a transposon has inserted into *aucE* (Table 3), probably disrupting the transcription of the operon. Two additional hybridization signals, corresponding to mRNAs of  $\sim 1.9$  and  $\sim 1.3$  kb, were also detected in all strains and are consistent with an independent transcription of either *aucF* and *aucG* or *aucG* alone, respectively. These data, together with the analysis of the pRJ9 sequence, suggest the presence of internal promoters in the operon, preceding both *aucF* and *aucG* (data not shown).

**Cloning of genes involved in immunity to aureocin A53.** Since *AucE*, *AucF*, and *AucG* are part of an ABC transporter which is associated to the membrane, these proteins may be toxic to the host cells under high concentrations. To avoid this problem, cloning experiments were attempted using plasmid pCC1, which contains the F plasmid single-copy origin of replication (*ori2*) and the inducible high-copy-number *oriV*. The 5.8-kb BamHI-EcoRI fragment of pRJ9 (Fig. 1) was cloned into plasmid pCC1, resulting in plasmid pRJ81. The toxicity of the insert to *E. coli* EPI300 cells was then investigated. Plasmid pRJ81 was induced to a high copy number per cell, and the growth of the host strain was analyzed and compared with that of the strains EPI300 and EPI300/pCC1 in a Bioscreen C system. However, no significant difference in



**FIG 3** (A) RT-PCR analysis of the *aucEFG* genes present on pRJ9. Lanes, 1 and 7, 100-bp DNA ladder (Fermentas Biolabs); lane 2, amplicon from *aucE* to *aucF* (381 bp) obtained from pRJ9 as the template; lane 3, amplicon from *aucE* to *aucF* obtained from cDNA; lane 4, RNA control; lane 5, blank (no cDNA); lane 6, amplification from *aucE* to *aucF* using plasmid pT181 as the template (negative control); lane 8, amplicon from *aucF* to *aucG* (366 bp) obtained from pRJ9; lane 9, amplicon from *aucE* to *aucF* obtained from cDNA; lane 10, blank (no cDNA). (B) RT-PCR analysis of the *orf11-orf10* genes present on pRJ9. Lane 1, internal control (*gyrA* amplicon; 447 bp) from cDNA obtained from strain A53; lane 2, negative control (*gyrA* amplification from RNA); lane 3, blank (no cDNA); lane 4, 100-bp DNA ladder (Promega); lane 5, amplification from *orf11* to *orf10* using plasmid pT181 as the template (negative control); lane 6, amplicon from *orf11* to *orf10* (576 bp) obtained from pRJ9 as the template; lane 7, amplicon from *orf11* to *orf10* obtained from cDNA; lane 8, RNA control; lane 9, blank (no cDNA).

growth rates was observed among the three strains tested (data not shown), indicating that the gene products of the 5.8-kb BamHI-EcoRI fragment of pRJ9 were not toxic to *E. coli*.

The BamHI-EcoRI insert was subcloned into the shuttle vector pELS100 to generate plasmid pRJ82. The immunity to aureocin A53 exhibited by the lactobacillus transformants carrying pRJ82 was then investigated (Fig. 4). The growth of the control strain *L. sakei* DSM20017/pELS100 was completely inhibited by 160 BU of aureocin A53. However, strain DSM20017/pRJ82 was not inhibited by any amount of aureocin A53 tested (up to 640 BU), demonstrating full immunity to aureocin A53. Strain DSM20017/pRJ82 was also able to produce aureocin A53 (160 BU ml<sup>-1</sup>). These results prove that the genetic determinants necessary for expression of immunity to aureocin A53 are located on the 5.8-kb BamHI-EcoRI fragment of pRJ9. The only intact genes found on this fragment are *aucA*, the bacteriocin structural gene (27), *orf10*, *orf11*, *aucE*, *aucF*, *aucG*, and *orf1*. *orf1* is a gene which encodes a protein involved in plasmid mobilization (10a). Therefore, its role in immunity was discarded.

Three-component ABC transporters, whose genetic organization is similar to that of the operon *aucEFG*, have been shown to

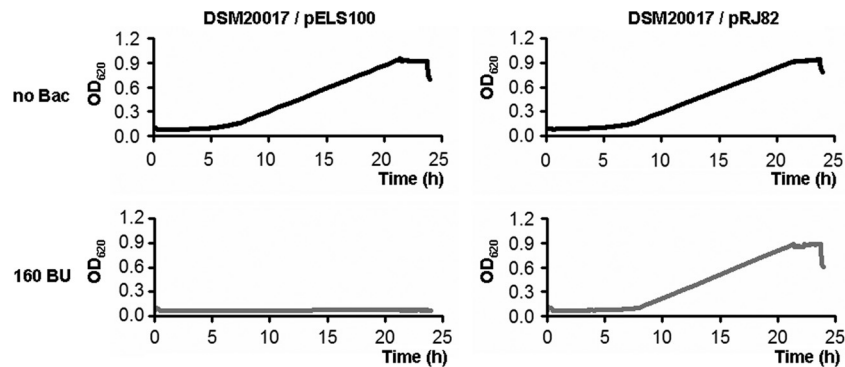


FIG 4 Growth curves exhibited by lactobacillus strains carrying either pELS100 or pRJ82 (5.8-kb BamHI-EcoRI fragment of pRJ9 cloned into pELS100) in the presence of 160 BU of aureocin A53 (gray curves). The controls (black curves) represent the growth of the strains in the absence of aureocin A53.

contribute to bacteriocin immunity by active extrusion of the peptide, reducing the concentration of the bacteriocin in direct contact with the cytoplasmic membrane (4, 23, 35, 41). To investigate if the ABC transporter encoded by *aucEFG* is involved in immunity to aureocin A53, a deletion of *aucG* was generated. Plasmid pRJ83 was then constructed by ligation of the HindIII-A fragment of pRJ9 to plasmid pELS100 (Fig. 1). pRJ83 could be transferred to *E. coli* but not to *L. sakei* DSM20017, in spite of the many attempts made to transform the latter strain with this recombinant plasmid derived from the same vector used for construction of pRJ82. The only major difference between pRJ83 and pRJ82 is the deletion, in the former plasmid, of *orf1* (involved in mobilization) and of about 90% of *aucG*. As in pRJ82, the aureocin A53 structural gene (*aucA*) remained intact in pRJ83. These results suggest that *AucG*, the largest membrane component of the ABC transporter, may play an important role in immunity to aureocin A53, which would explain the lack of cellular viability, in the presence of the bacteriocin, exhibited by the lactobacillus cells harboring this deletion mutant. Similar observations have been described in the literature. According to Rincé et al. (32), who studied genes involved in production of lactacin 481, the immunity to this lantibiotic was lost if any one of the genes *lctE*, *lctF*, or *lctG* was deleted.

To test if *aucEFG* are the only genes required for immunity to aureocin A53, an amplicon of 3,041 bp, including these three genes and also the putative promoter region, was cloned into the shuttle vector pNO6. The generated plasmid, pRJ85, was transferred to *S. aureus* RN4220, a strain sensitive to aureocin A53. The resultant transformant showed partial immunity to aureocin A53 (Fig. 5) since it exhibited a much longer lag phase in the presence of aureocin A53 than strain A53, the native host strain of pRJ9. The strain carrying the empty vector (pNO6) was not able to grow in the presence of aureocin A53. Taken together, these results suggest that other gene(s) present on pRJ9 may be required for full expression of immunity to aureocin A53.

In the insert present on plasmid pRJ82, which conferred full immunity to *L. sakei*, the products of *orf11* and *orf10* are the only other candidates to be involved in immunity. *orf11* and *orf10*, whose functions were unknown, are encoded in the opposite strand and in the same reading frame, with no overlapping and no intergenic region between them. RBS could also be found upstream of each *orf* although a canonical RBS was not found preceding any gene (data not shown). *orf11* starts with a TTG codon, while *orf10* starts with an ATG codon. A putative SigA-dependent

promoter is located upstream of *orf11*. A  $-10$  region, with five matches to the consensus sequence, and a  $-35$  region, with four matches to the consensus sequence, were found 35 bp and 58 bp, respectively, upstream of the start codon TTG of *orf11* (data not shown), with 17 bp between them. RT-PCR analysis (Fig. 3B) detected an amplicon corresponding to cotranscription of both *orf11* and *orf10* (lane 7), confirming that these genes are coordinately transcribed.

Orf11 and Orf10 share 80% and 61% similarity, respectively, with hypothetical proteins described in *Lactococcus lactis* that are not found in the gene clusters of other bacteriocins and whose functions are presently unknown. Orf10 shares common characteristics with proteins involved in bacteriocin immunity, especially with those from class II bacteriocins, since it possesses three transmembrane domains (data not shown), high pI (10.1), and 96 amino acids. On the other hand, Orf11 possesses 94 amino acids but does not present any transmembrane domain. Although anionic (pI of 4.9), Orf11 cannot be excluded as part of the immunity

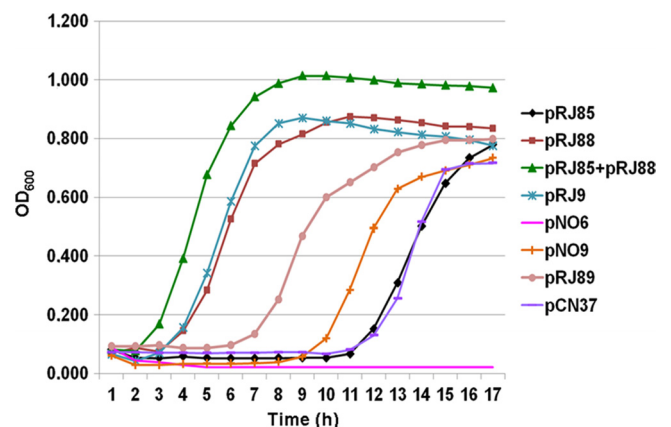


FIG 5 Growth curves of staphylococcal strains carrying different plasmids in the presence of aureocin A53 (2,048 BU). With the exception of pRJ9, whose host strain is A53, the remaining plasmids are in the genetic background of strain RN4220. pNO6 (pCC1 plus pIL252), pNO9 (pCC1 plus pT181), and pCN37 are the vectors used, and their host strains were employed as controls. pRJ85 represents *aucEFG* cloned into pNO6; pRJ88 represents *aucIB-aucIA* cloned into pNO9, and pRJ89 represents *aucIB-aucIA* cloned into pCN37. The curves were generated with the results representing the means of three independent experiments.



system since different mechanisms may operate for different bacteriocins and since aureocin A53 has features distinct from most class II bacteriocins (27). For this reason, a 735-bp amplicon containing both *orf11* and *orf10*, including the putative promoter, was cloned into pCN37, and the recombinant plasmid (pRJ89) (Fig. 1) was also transferred to *S. aureus* RN4220. However, again, only a partial immunity to aureocin A53 was exhibited by the transformants carrying pRJ89 (Fig. 5). The transformants exhibited a longer lag phase in the presence of aureocin A53 than strain A53 although their lag phases were shorter than the lag phase exhibited by the same host strain carrying the empty vector (pCN37).

To test if the presence of both operons *aucEFG* and *orf11-orf10* would result in a full-immunity phenotype, plasmids pRJ85 and pRJ88 (carrying *orf11-orf10* on pNO9) were sequentially introduced into strain RN4220 by transformations. The resultant strain harboring both plasmids exhibited immunity to aureocin A53 at an even higher level than that observed for strain A53, growing faster and reaching a higher cellular density in the presence of the bacteriocin (Fig. 5).

From the data presented in Fig. 5, it can also be observed that plasmid pRJ88 (carrying *orf11-orf10* on a pT181 derivative) confers a higher immunity to staphylococcal cells than plasmid pRJ85 (carrying *aucEFG* on a pIL252 derivative). The immunity conferred by pRJ88 was similar to that conferred by pRJ9, the native staphylococcal plasmid encoding aureocin A53. However, such observations may result from a gene dosage effect due to a much higher copy number of pT181*mcs* (about 100 copies/cell), a staphylococcal plasmid, than that of pIL252, a LAB plasmid, which appears to be maintained at very low copy number in a staphylococcal background (data not shown). This hypothesis was confirmed by the experiments performed with pRJ89, which carries the operon *orf11-orf10* cloned into pCN37, a staphylococcal shuttle plasmid whose copy number (15 to 20 copies/cell) is lower than that of pT181*mcs*. As shown in Fig. 5, the staphylococcal transformant carrying pRJ89 grew more slowly than the transformant carrying pRJ88 but still faster than the transformant harboring pRJ85. However, here again, these results may be explained by the high copy number of pCN37 in relation to that of pIL252 (data not shown).

To test if cloning of *aucEFG* into either pT181*mcs* or pCN37 would result in increased immunity to aureocin A53, attempts were made to construct pT181*mcs* or pCN37 derivatives carrying this operon. However, recombinant plasmids could never be recovered in four independent experiments performed with each vector. Therefore, these data suggest that under high concentrations the products encoded by these genes may be toxic to the staphylococcal host cells.

Taken together, these data suggest that the operon *orf11-orf10* is indeed required for full expression of immunity to aureocin A53, complementing the role played by *aucEFG*. Therefore, from here, *orf11* and *orf10* will be referred to as *aucIB* and *aucIA*, respectively.

The presence of more than one function involved in immunity to a given bacteriocin has already been described in other systems, such as in some lantibiotics and in cyclic bacteriocins (6, 24, 40). Lantibiotic-producing strains possess either both or just one of the self-protection systems LanFEG and LanI/LanH. LanFEG is observed for mersacidin, epidermin, lactacin 481, and mutacin II, while LanI can be found in the immunity system for epicidin 280, lactocin S, and Pep5 (6). In relation to subtilin and nisin, it has

been observed that the expression of either self-protection system confers partial immunity to these lantibiotics; however, the expression of both systems confers full immunity to them (37, 38).

In the case of nukacin ISK-1, a lantibiotic produced by *Staphylococcus warneri* ISK-1, the self-protection system against this bacteriocin is conferred by NukFEG and NukH (31). Like NukH, AucIA is a membrane protein with three transmembrane domains. However, in contrast to NukH and according to the TMpred program, AucIA could be inserted into the bacterial membrane with its N terminus oriented in the outer interface part of the cell membrane.

The immunity gene of most linear class II bacteriocins is located immediately downstream of, and in the same operon as, the bacteriocin structural gene (21, 29). Therefore, the location of the operon *aucIB-aucIA* downstream of, but on the opposite strand of, the aureocin A53 structural gene, *aucA*, is very unusual. However, there are other examples of atypical locations, such as the immunity genes of bacteriocins LsbA and LsbB, enterocin Q, and carnobacteriocin A, which are located next to the structural genes but have the opposite orientations (17, 18, 19).

**Analyses of pRJ9 mutants generated upon transposon insertion.** The exact position of the transposon insertion in each strain carrying a pRJ9 mutation was determined by sequencing and is presented in Table 3. Mutants MB139 (pRJ38) and MB140 (pRJ39) have a transposon inserted into *orf6* and *aucE*, respectively. Two other mutant strains, MB38 (pRJ14) and MB143 (pRJ40), have a transposon inserted into *orf8*.

All mutants showed a reduction in the amount of bacteriocin found in the culture supernatant (Table 3). The mutant affected in *orf6* exhibited a 32% reduction in the amount of aureocin A53 secreted. Orf6, a 61-amino-acid polypeptide, shares 100% identity to a hypothetical protein encoded by other *S. aureus* plasmids not encoding bacteriocin production. Therefore, Orf6 should not be directly involved in aureocin A53 production. Mutants affected in *orf8* exhibited the most drastic reduction, which corresponded to less than 3% of the aureocin A53 produced by the wild-type strain, suggesting that *orf8* is important for the maintenance of the normal levels of aureocin A53 secretion. Orf8, which seems to form an operon with *orf7*, shows about 50% similarity to a number of hypothetical membrane proteins found in many Gram-positive bacteria. However, additional studies are needed to investigate its role in aureocin A53 externalization.

Strain MB140, whose transposon is inserted into *aucE*, also exhibited a low level of bacteriocin secreted into the medium (a 76% reduction), suggesting that AucE also plays a role in the maintenance of the normal levels of aureocin A53 secretion. Since AucE is part of the unique ABC transporter encoded by the aureocin A53 gene cluster, this dedicated ABC transporter may also be involved in bacteriocin externalization, as found in other bacteriocin systems (6, 24, 40).

The mutant affected in *aucE* (pRJ39) exhibited a 16-fold increase in sensitivity to aureocin A53 compared to strain A53 (Table 3). The remaining mutants did not show differences in the immunity levels to exogenous aureocin A53 compared to the wild-type strain A53. Such results suggest that neither *orf6* nor *orf8* plays a role in the immunity phenotype and clearly implicate AucE in this phenotype, confirming the data presented above.

In conclusion, aureocin A53 is the first class II linear staphylococcal to have its immunity genes investigated. Our data show that this bacteriocin, differently from most class II bacteriocins



and similarly to various lantibiotics and cyclic bacteriocins, presents more than one system involved in the self-protection of its producer strain. One such system involves a dedicated ABC transporter which probably acts by active extrusion of the peptide. The second system involves an operon which encodes a hydrophobic cationic polypeptide, AucIA, which shares characteristics with immunity proteins generally associated to the membrane and found in the gene clusters of many class II bacteriocins, and an anionic protein, AucIB, never described before as part of a bacteriocin immunity system. Moreover, we found evidence of the major role played by a putative membrane protein, Orf8, in aureocin A53 externalization. The individual role played by AucIA and AucIB in immunity to aureocin A53 and the function of Orf8 in aureocin production and/or secretion are under investigation.

## ACKNOWLEDGMENTS

J.D.S.N., H.C., M.L.V.C., and L.R.F. were recipients of scholarships from CNPq/Brazil. This study was supported by grants from CNPq, FAPERJ, CAPES, and PRONEX to M.D.C.D.F.B.

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