The gene cluster of aureocyclicin 4185: the first cyclic bacteriocin of *Staphylococcus aureus*

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*Staphylococcus aureus* 4185 was previously shown to produce at least two bacteriocins. One of them is encoded by pRJ101. To detect the bacteriocin-encoding gene cluster, an ~9160 kb region of pRJ101 was sequenced. In silico analyses identified 10 genes (*aclX, aclB, aclI, aclC, aclD, aclA, aclF, aclG and aclH*) that might be involved in the production of a novel cyclic bacteriocin named aureocyclicin 4185. The organization of these genes was quite similar to that of the gene cluster responsible for carnocyclin A production and immunity. Four putative proteins encoded by these genes (*AclT, AclC, AclD and AclA*) also exhibited similarity to proteins encoded by cyclic bacteriocin gene clusters. Mutants derived from insertion of Tn917-lac into *aclC*, *aclF*, *aclH* and *aclX* were affected in bacteriocin production and growth. *AclX* is a 205 aa putative protein not encoded by the gene clusters of other cyclic bacteriocins. *AclX* exhibits 50 % similarity to a permease and has five putative membrane-spanning domains. Transcription analyses suggested that *aclX* is part of the aureocyclicin 4185 gene cluster, encoding a protein required for bacteriocin production. The *aclA* gene is the structural gene of aureocyclicin 4185, which shows 65 % similarity to garvicin ML. *AclA* is proposed to be cleaved off, generating a mature peptide with a predicted *M*<sub>r</sub> of 5607 Da (60 aa). By homology modelling, *AclA* presents four α-helices, like carnocyclin A. *AclA* could not be found at detectable levels in the culture supernatant of a strain carrying only pRJ101. To our knowledge, this is the first report of a cyclic bacteriocin gene cluster in the genus *Staphylococcus*.

INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins and are produced by a large variety of bacteria. They typically inhibit the growth of strains closely related to the producer strain although some bacteriocins exhibit a broad spectrum of activity, being able to inhibit different genera and species of bacteria (Heng *et al.*, 2007). In Gram-positive bacteria, bacteriocins are currently classified into four major classes (Heng *et al.*, 2007). Class I bacteriocins, or lantibiotics, are small, heat-stable peptides characterized by the presence of post-translationally modified amino acids (Bierbaum & Sahl, 2009). Class II bacteriocins are small, heat-stable peptides that do not contain modified amino acids (Nissen-Meyer *et al.*, 2009). Class III bacteriocins are heat-labile antimicrobial proteins (Heng *et al.*, 2007). Finally, class IV bacteriocins are cyclic peptides, formed by the post-translationally covalent linkage between their carboxy and amino termini (van Belkum *et al.*, 2011).

Staphylococcins are bacteriocins produced by strains belonging to the genus *Staphylococcus*. The staphylococcins so far described belong to classes I, II or III. Most are lantibiotics (Bastos *et al.*, 2009; Daly *et al.*, 2010). Very few staphylococcins, such as aureocin A70 (Netz *et al.*, 2001), aureocin A53 (Netz *et al.*, 2002) and epidermicin NI01 (Sandiford & Upton, 2012), are included in class II. Only one class III staphylococcin, lysostaphin, has been described (Bastos *et al.*, 2010) and, thus far, no staphylococcin belonging to class IV has been reported.

Class IV or cyclic bacteriocins are synthesized as linear precursor proteins, containing a leader peptide (2–35 aa

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Received 5 December 2013
Accepted 21 February 2014

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DOI 10.1099/mic.0.075689-0
residues) that is cleaved off during the maturation process (van Belkum et al., 2011). The linear peptide (35–70 aa residues) is cyclized by formation of an amide bond between the N and C termini, before being exported out of the cell. The enzymes that are responsible for cleavage of the leader peptide and ligation of the carboxy and amino termini have not yet been identified. The cyclic nature of these peptides renders them resistant to many proteases and provides great stability across a wide range of temperatures. Several genetic determinants are involved in production of cyclic bacteriocins and they are located on either the chromosome or plasmids (van Belkum et al., 2011). Although the number of genes among the gene clusters varies and similarity between the gene products of the various gene clusters is limited, they share a number of features. Most of the proteins encoded by these gene clusters contain multiple putative membrane-spanning domains and are provably associated with the membrane (van Belkum et al., 2011).

Cyclic bacteriocins permeabilize the membrane of susceptible cells, resulting in leakage of ions, dissipation of the membrane potential and cell death. Producer strains are protected from the bactericidal effects of their bacteriocins by cognate immunity systems, expression of which is invariably linked to bacteriocin production (Bierbaum & Sahl, 2009; Heng et al., 2007; Nissen-Meyer et al., 2009). Proteins involved in immunity to cyclic bacteriocins have a high pi and one or two putative membrane-spanning domains, which suggest that they are associated with the membrane. However, the presence of a dedicated immunity protein alone is not enough to confer full immunity. In addition, ABC transporters also play a role in immunity (van Belkum et al., 2011). Nevertheless, it is remarkable that not all gene clusters of cyclic bacteriocins encode these multi-component ABC transporters (van Belkum et al., 2011).

Cyclic bacteriocins have been described in Enterococcus faecalis (enterocin AS-48; AS-48), Lactobacillus gasseri (gassericin A), Lactobacillus reuteri (reutericin 6), Lactobacillus acidophilus (acidocin B), Clostridium beijerinckii (carnocycin A), Carnobacterium mairtaromaticum (carnocycin A; CcIA), Bacillus subtilis (subtilosin A), Butyrivibrio fibrisolvens (butyrivibricin AR10), Streptococcus uberis (uberolysin), Lactococcus spp. (lactocyclicin Q), Leuconostoc mesenteroides (leukocyclicin Q; LcyQ) and Lactococcus garvieae (garvicin ML; GarML) (Maqueda et al., 2008; Masuda et al., 2011; van Belkum et al., 2011).

In a previous study, we have shown that the antimicrobial activity exhibited by Staphylococcus aureus 4185, a strain involved in bovine mastitis, is due to production of more than one antimicrobial peptide, recovered with 40 and 80 % 2-propanol, respectively, during HPLC runs for peptide purification (Ceotto et al., 2010). The antimicrobial substances produced by Staphylococcus aureus 4185 display antagonistic activity against important food-borne pathogens, including Listeria monocytogenes (Ceotto et al., 2009), thus showing great potential as food preservatives, prompting us to investigate them further. In this study, we found out that one of the antimicrobial peptides produced by Staphylococcus aureus 4185 is encoded on the single plasmid, pRJ101, carried by this strain. An ~9160 kb region of pRJ101 was then sequenced and the in silico analyses performed revealed that it encompasses the whole gene cluster for a new staphylococcin, named aureocyclicin 4185 (AcA), which is related to cyclic bacteriocins. Although many bacteriocins from staphylococci have been described, this is the first that seems to be cyclic.

METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in the present study are listed in Table 1. Escherichia coli strains were cultivated at 37 °C in Luria–Bertani medium (LB; Difco) supplemented with 75 µg ampicillin ml–1 (Sigma), when appropriate. Staphylococcus aureus and Micrococcus luteus ATCC 4698 were grown at 37 °C in brain heart infusion medium (BHI; Difco), in trypticase soy broth (TSB; Difco) or in GM17 [M17 (Difco) supplemented with 0.5 % (w/v) glucose. Staphylococcus aureus strains carrying pRJ101 derivatives tagged with Tn917-lac were grown in the presence of erythromycin (Em; Sigma) at 10 µg ml–1. All media were supplemented with agar at 1.5 or 0.7 % (w/v), when required.

Staphylococcus aureus MB608 was created by mobilization of plasmid pRJ117 from Staphylococcus aureus MB497 to Staphylococcus aureus RN4220, using pGO1 as the conjugative plasmid, as described by Coelho et al. (2009).

Plasmid DNA isolation and manipulations. Isolation of Escherichia coli plasmid DNA (Table 1) was done with Qiang-tip 20 columns (Qiagen) according to the manufacturer’s instructions. The same kit was used to purify plasmid DNA from Staphylococcus aureus. In this case, the extractions were performed after treatment of the cells, for 30 min at 37 °C, with a lysis solution [50 mM Tris/HCl, pH 7.8, 40 mM EDTA and 25 % (w/v) sucrose] supplemented with 20 mg lysozyme ml–1 (Sigma), 1 mg lysostaphin ml–1 (Sigma), 100 µg RNase A ml–1 (Sigma) and 2.5 µg SDS ml–1.

Restriction enzymes (Invitrogen), T4 DNA-ligase (Invitrogen) and Taq DNA-polymerase (Fermentas) were used according to the manufacturers’ recommendations. Oligonucleotides (Tables S1 and S2, available in the online Supplementary Material) were purchased from Invitrogen. DNA preparations were quantified by using Qubit 2.0 (Invitrogen).

Transposon mutagenesis. Mutants of plasmid pRJ101 were generated by transposon mutagenesis with Tn917-lac using plasmid pTV32T as the delivery vector. To do so, plasmid pTV32T was transduced to strain Staphylococcus aureus 4185 at 32 °C using phage 80a as the transducing phage (Bastos et al., 1980), creating strain MB467. The mutagenesis experiments were then performed as described by Oliveira et al. (1998). The exact position of the transposon insertion into plasmid pRJ101 was determined by DNA sequencing, using an 18 bp primer (5’-TGTAAGCTTACCGACTAATGAG-3’), the 3’ end of which is located 101 bp upstream of the left end of the transposon (Netz et al., 2001). Bacteriocin production and immunity to the bacteriocins produced by Staphylococcus aureus 4185 expressed by these mutants were tested as described below.

Plasmid curing experiments. Strain MB497, the host strain of plasmid pRJ117, a pRJ101 derivative tagged with Tn917-lac but not affected in bacteriocin production, was used for curing experiments performed as described by Nascimento et al. (2012), except for the curing temperature, which was 43 °C. Plasmid DNA was isolated.
from Em\(^R\) colonies and subjected to electrophoresis in 0.7% (w/v) agarose gel to confirm plasmid loss. Bacteriocin production and immunity exhibited by the plasmid-cured derivatives to the bacteriocins produced by Staphylococcus aureus 4185 were also investigated as described below.

**Assay for bacteriocin production.** The investigation of bacteriocin production was performed on GM17 medium at 37 °C with the plasmid-cured derivatives and strain 4185, using the deferred-antagonism assay as described by Giambiagi-Marval et al. (1990). *M. luteus* ATCC 4698, which is highly sensitive to staphylococcins, was used as the target strain (Bastos et al., 2009). Additionally, the same test was performed on BHI medium at 32 °C with *Staphylococcus aureus* mutants and strains MB493 and 4185. These last two strains were tested at 37 °C as well. Analysis of the results was made using the ‘real inhibition zone’, which was calculated by subtracting the diameter of the spot formed by the growth of the producer strain from the diameter of the inhibition zone. According to their ‘real inhibition zone’, the strains were classified as negligible-producer (5 mm), based on statistical analyses (P<0.05). For example, the spot diameter formed by strain Z was 8 mm and the diameter of the inhibition zone was 10 mm. The ‘real inhibition zone’ of the strain Z was 2 mm. Therefore, this strain was classified as a weak-producer strain. These experiments were repeated three times.

To test for immunity to the bacteriocins produced by *Staphylococcus aureus* 4185, the same assay was used, employing this strain as the bacteriocin-producer and either the *Staphylococcus aureus* 4185 mutants or the plasmid-cured derivatives as the target microorganisms. BHI medium was used in this test.

**Cloning of the HindIII fragments of pRJ101.** Digestion of plasmid pRJ101 with HindIII generates five DNA fragments of approximately 3.9, 3.7, 2.5, 0.8 and 0.7 kb. Each fragment was individually cloned into pBluescript SK\(^+\) (Stratagene). The recombinant plasmids generated upon ligation were transformed into thermal-competent *Escherichia coli* DH5\(\alpha\). Ampicillin-resistant transformants were chosen for plasmid DNA isolation, followed by digestion with HindIII. Recombinant plasmids carrying HindIII fragments A (~3.9 kb) and B (3.7 kb) were chosen for DNA sequencing. No recombinant plasmids carrying either the HindIII-D or -E fragments were recovered in the single experiment performed.

**pRJ101 DNA sequencing.** Plasmid DNA templates were prepared strictly following the protocol suggested by Applied Biosystems. Automated sequencing was performed using the ABI Prism 3100 System and the Terminator Chemistry Big Dyes, version 3.1 (Applied Biosystems). Primers were used according to the plasmids and inserts used as templates (Table S1). When plasmid pBluescript SK\(^+\) was

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**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>Restriction-deficient derivative of <em>Staphylococcus aureus</em> NCTC8325</td>
<td>Kreiswirth et al. (1983)</td>
</tr>
<tr>
<td>RN4220</td>
<td>Bac(^+) + + +, pRJ101; Bac(^R)</td>
<td>Cetto et al. (2009)</td>
</tr>
<tr>
<td>4185</td>
<td>Bac(^+) + + +, pTV32T; Cm(^R); Em(^R)</td>
<td>This work</td>
</tr>
<tr>
<td>MB467</td>
<td><em>Staphylococcus aureus</em> 4185; pTV32T; Cm(^R); Em(^R)</td>
<td>This work</td>
</tr>
<tr>
<td>MB493</td>
<td><em>Staphylococcus aureus</em> 4185 cured of pRJ101; Bac(^+); Bac(^R)</td>
<td>This work</td>
</tr>
<tr>
<td>MB495</td>
<td><em>Staphylococcus aureus</em> 4185; pRJ101 accL::Tn917-lac (-20.3 kb); Em(^R); Bac(^+)</td>
<td>This work</td>
</tr>
<tr>
<td>MB497</td>
<td><em>Staphylococcus aureus</em> 4185; pRJ117 (-20.3 kb); Em(^R); Bac(^+) + + +</td>
<td>This work</td>
</tr>
<tr>
<td>MB558</td>
<td><em>Staphylococcus aureus</em> 4185; pRJ101accL::Tn917-lac (-20.3 kb); Em(^R); Bac(^+)</td>
<td>This work</td>
</tr>
<tr>
<td>MB576</td>
<td><em>Staphylococcus aureus</em> 4185; pRJ101accH::Tn917-lac (-20.3 kb); Em(^R); Bac(^-)</td>
<td>This work</td>
</tr>
<tr>
<td>MB594</td>
<td><em>Staphylococcus aureus</em> 4185; pRJ101accC::Tn917-lac (-20.3 kb); Em(^R); Bac(^-)</td>
<td>This work</td>
</tr>
<tr>
<td>MB127</td>
<td>pTV32T; Em(^R); Cm(^R)</td>
<td>Oliveira et al. (1998)</td>
</tr>
<tr>
<td>MB608</td>
<td><em>Staphylococcus aureus</em> RN4220; pRJ117</td>
<td>This work</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5(\alpha)</td>
<td>lacZaM15; hsdR(^-)</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>pBluescript SK(^+); Ap(^R)</td>
<td>Bullock et al. (1987)</td>
</tr>
<tr>
<td>EC202</td>
<td>DH5(\alpha); pBluescript SK(^+)::HindIII-ApRJ101 (~6.9 kb)</td>
<td>This work</td>
</tr>
<tr>
<td>EC204</td>
<td>DH5(\alpha); pBluescript SK(^+)::HindIII-BpRJ101 (6.7 kb)</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRJ101</td>
<td>Encodes aureocyclicin 4185 (~11.7 kb)</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ117</td>
<td>pRJ101::Tn917-lac (~20.3 kb); Em(^R); Bac(^+) + +</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ118</td>
<td>pRJ101 accL::Tn917-lac (~20.3 kb); Em(^R); Bac(^+)</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ120</td>
<td>pRJ101accH::Tn917-lac (~20.3 kb); Em(^R); Bac(^+)</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ121</td>
<td>pRJ101accC::Tn917-lac (~20.3 kb); Em(^R); Bac(^-)</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ122</td>
<td>pRJ101accC::Tn917-lac (~20.3 kb); Em(^R); Bac(^-)</td>
<td>This work</td>
</tr>
<tr>
<td>pTV32T</td>
<td>Carries Tn917-lac (12.4 kb); Cm(^R); Em(^R)</td>
<td>Perkins &amp; Youngman (1986)</td>
</tr>
<tr>
<td>pBluescript SK(^+)</td>
<td>3.0 kb; Ap(^R)</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

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used as the cloning vector, primers M13 forward and reverse were used to sequence the ends of each insert. Finishing methods included primer walking, PCR-based techniques and direct sequencing from pRJ101. Both strands were sequenced independently. DNA sequencing was performed by the Laboratório de Unidade Genômica of the Instituto de Biofísica Carlos Chagas Filho, UFRJ, Rio de Janeiro. A unique contig was assembled by using SeqMan (DNASTAR-Lasergene, version 7.0.0).

**In silico sequence analyses.** The nucleotide sequence obtained was searched for potential ORFs in all six possible frames by using the program ApE, version 2.0.46 (http://biologylabs.utah.edu/jorgensen/wayned/apen). The same program was used to generate the genetic map of pRJ101. The BlastP program was used to search for sequence similarities between the predicted amino acid sequences of each ORF and other proteins available in GenBank. The presence of putative transcription terminator sites was investigated using the Kinofold server available at http://kinfold.cufe.fr/cgi-bin/form.pl.

Protein transmembrane helices were predicted by using the TMpred Server (http://www.ch.embnet.org/software/TMPRED_form.html) and PsiPred (http://bioinf.cs.ucl.ac.uk/psipred). With the PsiPred software, it is possible to obtain diagrams that show cartoons of the transmembrane helix predictions and a prediction of pore-lining helices. TMpred makes a prediction of membrane-spanning regions and their orientation. To detect putative α, β promoters upstream of each ORF, programs PPP (http://bioinformatics.biol.rug.nl/webscript/PPP/start.php) and BPROM (http://linux1.softberry.com) were used. The program PBL (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_bth.html) was used to detected helix-turn-helix motifs. Finally, the program ProtParam (http://web.expasy.org/compute_pi/) was used to predict pl and M_r.

**Homology modelling.** The sequence of AclA was submitted to the Swiss-model automated modelling server (Guex & Peitsch, 1997) to derive a 3D model. The study was assessed with the analytical tools also available in the Pymol software and with the programs in the Procheck suite for model validation (Laskowski et al., 1993). The molecular model of AclA was submitted to model refinement and energy minimization conducted with the GROMOS96 v.43B1 force field implemented in the SwissPDB viewer v.3.7b2 and online databases. The final refined theoretical structure of the peptide was achieved by a harmonic constraint. Additional refinements of the model were performed using a similar minimization protocol in which the types of residues constrained during the minimization were varied: initially, only residues of the most favoured regions of the Ramachandran plot were allowed to move, and then residues with a high model B-factor and/or force field energy were included. Structure refinement was based on the CxIA 3D structure (2kJF, www.pdb.org).

**Growth kinetics of the pRJ101::Tn917-lac mutants and strains 4185 and MB493.** The strains and mutants were grown on solid BHI medium for 18 h at 37 °C. Thereafter, two colonies of each mutant analysed were gently removed from the medium with the aid of a sterile needle and inoculated into 3 ml of BHI broth. The cultures were homogenized and 200 μl was transferred to a 96-well polystyrene microtitre plate (TPP 92096). Growth curves were monitored at 37 °C in a SpectraMax Plus384 Absorbance Microplate Reader (Molecular Devices), which measured the culture OD600 at time intervals of 1 h, for 18 h. Four replicas of the experiment were performed for each mutant and each experiment was repeated twice. Graphs originated from these measurements were generated using Excel software (version 2007).

**Transcription analyses.** The isolation of total RNA from either Staphylococcus aureus 4185, MB493 (cured of pRJ101) or MB495, a mutant generated by transposition of Tn917-lac into pRJ101, was performed with an RNeasy Mini kit (Qiagen), following the adapted protocol described elsewhere (Coelho et al., 2009). RNA preparations were quantified by using Qubit. DNase I-pretreated RNA (55 ng) was subjected to cDNA synthesis using a Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas) as described by the manufacturer. RT-PCR analyses of aclA, the bacteriocin structural gene, were performed with primers pRJ101-4185A-F and pRJ101-4185A-R (Table S2). Those of aclX were performed with primers pRJ101-SSA-F and pRJ101-SSA-R, and those from part of aclX to aclB with primers pRJ101-b3F and pRJ101-b4R (Table S2). An internal control was performed using primers GyrF and GyrR (Table S2) targeting the housekeeping gene gyrA, which codes for DNA-glyrase subunit A.

The PCR mixtures consisted of 1× reaction buffer, 0.25 U of Taq DNA-polymerase (Fermentas), 1.5 mM MgCl₂, 0.2 mM of each dNTP (Promega) and 20 pmol of each primer. The PCRs were performed as follows: an initial denaturation step at 92 °C for 3 min; 30 cycles at 92 °C for 1 min, the annealing temperature for 1 min (which varied depending on the pair of primers) and 72 °C for 1 min; with a final extension step at 72 °C for 5 min. The amplicons were analysed on 1.4% (w/v) agarose gels, using the 100 bp DNA ladder O’RangeRuler (Fermentas) as size markers.

**Statistical analyses.** A t-test was used to determine if the differences in OD readings in the growth kinetics experiments were significant (P<0.05). For these analyses, Excel 2007 was used. To determine if the differences in the real inhibition zones obtained in the experiments of bacteriocin production were significant, statistical analyses were performed with two-way ANOVA method, using the Graph Pad Prism (version 5.0) program. Post-hoc statistical tests were performed using the Bonferroni method. P<0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSION**

**Curing of plasmid pRJ101**

Previous studies have suggested that strain 4185 produces more than one antimicrobial peptide (Ceotto et al., 2010). To test if some of these substances was plasmid encoded, curing experiments were performed with strain MB497, the host strain of plasmid pRJ117, a pRJ101 derivative tagged with Tn917-lac but not affected in bacteriocin production (see below). One colony that had lost pRJ117 was selected and named MB493. Bacteriocin production by strains 4185 and MB493 was tested on solid GM17 medium at 37 °C. As expected, strain MB493 exhibited smaller inhibition zones than strain 4185 against M. luteus ATCC 4698. When tested on solid BHI medium, no bacteriocin production was detected for strain MB493 against the same target micro-organism (Table 2). These results confirmed that the genes encoding one of the bacteriocins produced by strain 4185 were located on plasmid pRJ101. Moreover, they suggest that production of the chromosomally encoded bacteriocins produced by strain 4185 is being stimulated on GM17 medium. As the goal of this work was to study the bacteriocin encoded on pRJ101, BHI medium was chosen for all analyses involving the pRJ101::Tn917-lac mutants (see below).

The temperature used to grow the bacteriocin-producing strain is known to influence staphylococcin production positively or negatively (Nascimento et al., 2004). Therefore,
Table 2. Bacteriocin production by pRJ101::Tn917-lac mutants and by strains 4185 and MB493

Numbers represent the means ± SD of the real inhibition zones (mm; equal to the diameters of the inhibition zone minus the diameters of the producing strain growth spot), measured in three independent experiments. -, Negligible-producer strains (real inhibition zone ≤ 1.3 mm), +, weak-producer strains (1.3 mm < real inhibition zone ≤ 2.0 mm), ++, moderate-producer strains (2.0 mm < real inhibition zone ≤ 5 mm); +++, strong-producer strains (real inhibition zone > 5 mm). ND, Not determined.

<table>
<thead>
<tr>
<th>Producer strain</th>
<th>Temperature</th>
<th>Category</th>
<th>37 °C</th>
<th>32 °C</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM17 medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4185</td>
<td>7.67 ± 0.44</td>
<td>++</td>
<td></td>
<td>6.67 ± 0.58</td>
<td>+++</td>
</tr>
<tr>
<td>MB493</td>
<td>2.5 ± 0.44</td>
<td></td>
<td></td>
<td>1.67 ± 0.58</td>
<td>+</td>
</tr>
<tr>
<td>BHI medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4185</td>
<td>4.00 ± 1.00</td>
<td>+++</td>
<td>6.67 ± 0.58</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>MB493</td>
<td>0.00 ± 0.00</td>
<td>-</td>
<td>0.00 ± 0.00</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MB495</td>
<td>ND</td>
<td>ND</td>
<td>1.67 ± 0.58</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MB558</td>
<td>ND</td>
<td>ND</td>
<td>2.00 ± 1.00</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MB576</td>
<td>ND</td>
<td>ND</td>
<td>1.00 ± 1.00</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MB594</td>
<td>ND</td>
<td>ND</td>
<td>0.67 ± 1.15</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

The assays for bacteriocin production were repeated on solid BHI medium at 32 °C with strains 4185 and MB493. Taking together all experiments performed and using the criteria described in Methods, the wild-type strain 4185 was classified as a moderate-producer when grown at 37 °C, but as a strong-producer at 32 °C (Table 2). These results showed that growth at 32 °C stimulates bacteriocin production by strain 4185 and therefore only this temperature was used in the tests with the mutants.

MB608 is a derivative of strain RN4220 carrying plasmid pRJ117. This strain was constructed and used in bacteriocin assays to avoid the influence of the bacteriocins encoded on the chromosome of strain 4185 on the results. MB608 gave rise to inhibition zones against *M. luteus* on solid BHI medium. Attempts to detect bacteriocin production by strain MB608 in broth were then performed using both media (BHI and GM17) and growth temperatures (37 and 32 °C), but no inhibitory activity against *M. luteus* was observed in the culture supernatants (data not shown). Therefore, the bacteriocin encoded by pRJ101 does not seem to be present in the culture supernatant at detectable levels. Such observation precluded its purification, the determination of its molecular mass and amino acid sequencing.

A test was also performed to investigate if strain MB493 became sensitive to the bacteriocin encoded by pRJ101. The results showed that strain MB493 was not inhibited on solid medium by the bacteriocins produced by strain 4185 at both temperatures tested (37 and 32 °C), which suggests that strain 4185, and therefore MB493, is resistant to the bacteriocin encoded by pRJ101. These results precluded the investigation of the immunity exhibited by the pRJ101::Tn917-lac mutants.

Sequence analysis

Plasmid DNA sequencing was then performed to identify the bacteriocin gene cluster found on pRJ101. Different methods, which included cloning of HindIII fragments, primer walking, PCR-based techniques and direct sequencing from pRJ101, were employed. However, despite numerous attempts using different approaches, a small region of pRJ101 could not be sequenced. It is believed that this region contains a secondary structure with a long inverted repeat able to form a complex secondary structure, as found in other cyclic bacteriocin gene clusters (Diaz et al., 2003; Kalmokoff et al., 2003; Kawai et al., 2003; Kemperman et al., 2003; Martínez-Bueno et al., 1994; Wirawan et al., 2007; Zheng et al., 1999). The size of this region was estimated by PCR to be approximately 100 bp (data not shown). A unique sequence containing ~9160 bp was assembled with the HindIII fragments B, D, E and most of A, in this order (Fig. 1). The DNA sequence of the HindIII-C fragment, which lies between the HindIII-A and HindIII-B fragments, although determined (2538 bp), was not included in the present study as it does not carry any function involved in bacteriocin production.

The DNA sequence was then translated for ORF identification. This analysis revealed 10 putative ORFs encoded on the same DNA strand (aclX, aclB, aclI, aclT, aclC, aclD, aclA, aclF, aclG and aclH). The characteristics of the proteins encoded by these ORFs are listed in Table 3 and all of them were putatively linked to a cyclic bacteriocin production based on further analysis. The organization of the ORFs was quite similar, but not identical, to the organization of the gene cluster responsible for CclA production and immunity (van Belkum et al., 2010). This novel bacteriocin was named aureocyclicin 4185 (AclA). Therefore, the ORFs were referred to with the three letters ‘acl’ to follow the designation of the genes belonging to the CclA gene cluster (Fig. 1 and Table 3).

An ORF located in the bacteriocin gene cluster, aclA, encodes a putative peptide of 64 aa with 65% similarity to the pre-peptide of GarML, a cyclic bacteriocin produced by
Lactococcus garvieae (Table 3). This peptide is therefore believed to be the precursor protein of aureocyclicin 4185, the first cyclic bacteriocin to be reported in the genus Staphylococcus.

The cyclic bacteriocins are translated with a leader peptide in the amino terminus, which is cleaved off during a processing step to give rise to the mature peptide (van Belkum et al., 2011). As aureocyclicin 4185 could not be purified, to deduce the processing site in the AclA precursor, a comparison was made with the sequences of the precursor peptides of the cyclic bacteriocins GarML, LcyQ and CclA (Fig. 2a). Based on this comparison and taking into account that in most cyclic bacteriocins Leu or Ile is at the end of the N terminus (van Belkum et al., 2011), it is proposed that aureocyclicin 4185 is cleaved off between the fourth (Glu) and fifth (Leu) amino acid residues, generating a putative mature peptide of 60 aa and having a predicted leader peptide formed by the amino acid sequence MLLE (Fig. 2a). Such short leader peptides have already been observed for the cyclic bacteriocins CclA (Martin-Visscher et al., 2008), circulin A (Kemperman et al., 2003), GarML (Borrero et al., 2011), lactocyclic Q (Sawa et al., 2009), LcyQ (Masuda et al., 2011), subtilosin A (Zheng et al., 1999) and uberolyisin (Wirawan et al., 2007), which contain leader peptides of 4, 3, 3, 2, 2, 8 and 6 aa, respectively. In contrast, the cyclic antibacterial peptides AS-48 (Samyn et al., 1994), butyrivibrioic AR10 (Kalmokoff et al., 2003) and gassericin A (Kawai et al., 1998) contain longer leader peptides of 35, 22 and 33 aa, respectively. All further analyses regarding aureocyclicin 4185 were performed with the 60 aa putative mature peptide. This peptide has a predicted $M_r$ of 5607 Da and an estimated $\pi$ of 10.00. Additionally, in silico analyses suggest that AclA contains four β-helices enclosing a compact hydrophobic core. Based on these analyses, the region between amino acids 26 and 41 should be inserted into the membrane, being responsible for the putative pore formation activity of the peptide (data not shown).

Except for AclT and AclG, all other proteins encoded by the aureocyclicin 4185 gene cluster also contain putative membrane-spanning domains and might therefore be associated with the membrane. Four putative proteins (AclB, AclT, AclC and AclD) exhibited similarity to proteins encoded by the CclA gene cluster (Table 3). Three putative proteins (AclF, AclG and AclH) exhibited similarity to multicomponent ABC transporters, often present in the cyclic bacteriocin gene clusters and also involved in immunity (van Belkum et al., 2011). AclT and AclG both contain an ATP-binding domain (GxxGxGKST) and AclG also contains an ABC transporter signature motif (LSGGQ), indicating that these proteins are indeed part of ABC transporters (Davidson & Maloney, 2007).

A gene, cclH, found in the CclA gene cluster encodes a permease, part of a multicomponent ABC transporter (van Belkum et al., 2010). The corresponding gene found in the aureocyclicin 4185 gene cluster, aclH, showed 96% similarity to a multicomponent ABC transporter of Staphylococcus aureus (Table 3). Another gene, aclL, encodes a membrane component of the CclA transport machinery (van Belkum et al., 2010), but no gene product with similarity to CclE was encoded by the aureocyclicin 4185 gene cluster (Table 3). However, the gene product of aclX might play the role of CclE during aureocyclicin 4185 production.

AclX is not found among proteins encoded by the gene clusters of other cyclic bacteriocins based on amino acid sequence similarity (Maqueda et al., 2008; van Belkum et al., 2011). The aclX gene encodes a putative 205 aa (23.28 kDa) protein that exhibited 30% similarity to a Bacillus bataviensis permease (Table 3). AclD has an estimated $\pi$ of 9.34 and five β-helices, which could function as putative transmembrane domains. AclX may therefore be involved in aureocyclicin 4185 externalization, replacing CclE in the transport machinery. Moreover, aclB, the function of which is unknown, is the first gene of the CclA gene cluster, while, in the AclA gene cluster, aclX is
Table 3. Characteristics of the ORFs and the putative proteins encoded by the aureocyclicin 4185 gene cluster

<table>
<thead>
<tr>
<th>Predicted gene</th>
<th>Localization (gene size in bp)</th>
<th>Putative RBS</th>
<th>Putative promoter</th>
<th>Putative protein</th>
<th>Amino acids</th>
<th>Similarity* (reference)</th>
<th>Putative protein function/characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>aclX</td>
<td>1041–1658 (618)</td>
<td>AAGAGG</td>
<td>TTGGTT AATAAT</td>
<td>AclX</td>
<td>205</td>
<td>Permease of <em>Bacillus bataviensis</em> (50 %) (NF)</td>
<td>Permease/putative membrane protein</td>
</tr>
<tr>
<td>aclB</td>
<td>1756–3123 (1368)</td>
<td>AAGAAA</td>
<td>TTGAGT CATAAT</td>
<td>AclB</td>
<td>455</td>
<td>CclB of <em>Carnobacterium maltaromaticum</em> (53 %) (van Belkum et al., 2010)</td>
<td>Unknown/putative membrane protein</td>
</tr>
<tr>
<td>acll</td>
<td>3110–3292 (183)</td>
<td>GGGAGG</td>
<td>TTGAAA TTTTAT</td>
<td>Acll</td>
<td>60</td>
<td>NF</td>
<td>Immunity protein</td>
</tr>
<tr>
<td>aclT</td>
<td>3316–3945 (630)</td>
<td>AGGATG</td>
<td>TTGGTA TATTTA</td>
<td>AclT</td>
<td>209</td>
<td>CclT of <em>Carnobacterium maltaromaticum</em> (63 %) (van Belkum et al., 2010)</td>
<td>ATP-binding protein</td>
</tr>
<tr>
<td>aclC</td>
<td>3969–4472 (504)</td>
<td>AGGAGG</td>
<td>TTGAAT TCTTTT</td>
<td>AclC</td>
<td>167</td>
<td>CdIC of <em>Carnobacterium maltaromaticum</em> (53 %) (van Belkum et al., 2010)</td>
<td>Protein involved in biosynthesis and immunity (member of the DUF95 family)/putative membrane protein</td>
</tr>
<tr>
<td>aclD</td>
<td>4488–4991 (504)</td>
<td>AGGAGA</td>
<td>NF NF</td>
<td>AclD</td>
<td>167</td>
<td>CdID of <em>Carnobacterium maltaromaticum</em> (56 %) (van Belkum et al., 2010)</td>
<td>Unknown/putative membrane protein</td>
</tr>
<tr>
<td>aclA</td>
<td>5023–5217 (195)</td>
<td>GGAGG</td>
<td>TTGAAA TTTTAT</td>
<td>AclA</td>
<td>64</td>
<td>Garvicin ML of <em>Lactococcus garvieae</em> DCC43 (65 %) (Borrero et al., 2011)</td>
<td>Aureocyclicin 4185 precursor</td>
</tr>
<tr>
<td>aclF</td>
<td>5378–6481 (1104)</td>
<td>AGGAGG</td>
<td>? ?</td>
<td>AclF</td>
<td>367</td>
<td>Putative protein of <em>Staphylococcus xylosus</em> (78 %) (Planchon et al., 2007)</td>
<td>Multicomponent ABC transporter/putative membrane protein</td>
</tr>
<tr>
<td>aclG</td>
<td>6478–7158 (681)</td>
<td>GAGGTTG</td>
<td>NF NF</td>
<td>AclG</td>
<td>226</td>
<td>Putative protein of <em>Staphylococcus aureus</em> (92 %) (NF)</td>
<td>Multicomponent ABC transporter/ATP-binding protein</td>
</tr>
<tr>
<td>aclH</td>
<td>7155–8330 (1179)</td>
<td>AAGAGG</td>
<td>TCGAAA TATTAT</td>
<td>AclH</td>
<td>392</td>
<td>BacI-like protein of <em>Staphylococcus aureus</em> (96 %) (NF)</td>
<td>Multicomponent ABC transporter/putative membrane protein</td>
</tr>
</tbody>
</table>

*Analyses performed using BLASTP.
located upstream of aclB. AclB is a putative membrane protein.

The aclI gene encodes a putative peptide with no similarity to any previously described protein (Table 3). The putative 60 aa (7.2 kDa) protein has an estimated pI of 10.02 and two putative membrane-spanning domains, characteristics exhibited by dedicated immunity proteins encoded by cyclic bacteriocin gene clusters (van Belkum et al., 2011). These features exhibited by AclI suggest that this peptide may be involved in bacteriocin immunity. The membrane topology analysis performed with AclI suggests that its second α-helix is inserted into the membrane (data not shown).

At least one putative σ^{A}-dependent promoter is located upstream of seven genes found in the AclA gene cluster (Fig. 1; Table 3). In the regions upstream of the genes aclD and aclG no putative promoters were found. Additionally, it was not possible to determine the presence of a promoter upstream of aclF, as the upstream region of this gene is part of the non-sequenced small region (Table 3). All 10 genes detected were preceded at appropriate distances by a candidate RBS (Table 3). An inverted repeat of 42 bp resembling a putative rho-dependent stem-and-loop transcriptional terminator [ΔG = -20.7 kcal mol^{-1} (≈-86.6 kJ mol^{-1})] was detected 96 bp downstream of aclH, the last gene of the cluster (Fig. 1).

Modelling the 3D structure of AclA

The 3D structure of AclA was determined by molecular modelling, using the CclA 3D structure obtained by NMR (Martin-Visscher et al., 2009), as a refinement model. The analyses suggest that AclA, like CclA, folds into a compact globular bundle consisting of four conserved α-helices (Fig. 2b, c) enclosing a compact hydrophobic core. The head-to-tail fusion point should also be located in helix 4 (Fig. 2b, c). Moreover, the predicted structure of AclA shows a cluster of basic amino acid residues, such as Lys^{46}, Lys^{50}, Lys^{54} and Lys^{55} (Fig. 2a), distributed in the region of helices 3 and 4, that may confer a highly localized positive charge on the surface of the peptide. This feature has already been described in the structures of the cyclic peptides AS-48, CclA, circularin A, lactocyclicin Q, GarML and uberolysin (Borrero et al., 2011; Martin-Visscher et al., 2008). For these bacteriocins, it is believed that, at neutral pH, the localized Lys side chains would be protonated, imparting a localized, positive charge on the molecule. In the case of CclA, the net positive charge is probably a key component in attracting the cyclic bacteriocin to the anionic lipid membrane, thus facilitating membrane insertion (Martin-Visscher et al., 2009). Given that the predicted structure of AclA resembles that of other cyclic bacteriocins, we believe that its mechanism of action would be similar to that of the other cyclic bacteriocins, with the highly positive localized charge conferred by its four Lys residues aiding in AclA attachment to the membrane, prior to its insertion and pore formation.

Tn917-lac insertion mutants: bacteriocin production and growth kinetics

To investigate whether aclX is part of the AclA gene cluster, transposon mutagenesis was carried out employing Tn917-lac (which confers an Em^{R} phenotype). Five different pRJ101 mutants were obtained. The precise location of the Tn917-lac insertion was determined by DNA sequencing and is shown in Fig. 1. One mutant, MB495 (pRJ118; aclX::Tn917-lac), had the transposon inserted between the aclX start codon and its putative promoter (Table 1 and Fig. 1). Moreover, three other different mutants in the AclA gene cluster were obtained (Table 1 and Fig. 1): MB558 (pRJ120; aclF::Tn917-lac), MB576 (pRJ121; aclH::Tn917-lac) and

![Fig. 2. (a) Pre-peptide sequences of the cyclic bacteriocins garvicin ML (GarML), leucocyclicin Q (LcyQ), carnocyclin A (CclA) and aureocyclicin 4185 (AclA). The horizontal arrows indicate the cleavage site of the leader peptide. The vertical arrows indicate the Lys residues. (b) 3D model structure of aureocyclicin 4185 and (c) 3D model structure of carnocyclin A.](image-url)
MB594 (pRJ122; aclC::Tn917-lac). In mutant MB497, the strain used in the curing experiments, Tn917-lac was inserted 230 bp downstream of the termination codon of aclH, outside the AclA gene cluster (Fig. 1).

The aclF gene seems to be a hot spot for Tn917-lac insertion as 11 additional mutants had the transposon inserted exactly in the same position of mutant MB558. Bacteriocin production by each mutant was then investigated by the deferred-antagonism assay at 32 °C. Two mutants (MB495 and MB558) were classified as weak-producers, and two mutants (MB576 and MB594) and strain MB493 were classified as negligible-producers (Table 2). These results suggest that the Tn917-lac insertion upstream of aclX and into aclC, aclF and aclH affected the bacteriocin production by all these mutants. Therefore, the products of aclX, aclC, aclF and aclH are required for AclA production. By contrast, mutant MB497 was not affected in bacteriocin production, being considered a strong-producer.

The growth kinetics of strain 4185 and the four mutants MB495, MB558, MB576 and MB594 were investigated in BHI medium at 37 °C (Fig. 3). Except for mutant MB594 and the wild-type strain 4185, all other strains showed a significant reduction in their OD values at the end of the experiment (P<0.05; Table S3). In the case of mutants MB558 (aclF::Tn917-lac) and MB576 (aclH::Tn917-lac), the affected genes are part of a putative operon (aclFGH), possibly involved in bacteriocin externalization and immunity. The as-48EFGH operon encodes an ABC transporter that is related to higher immunity against exogenously added enterocin AS-48 and involved in self-protection against this bacteriocin. While As-48G is the ATP-binding subunit, the membrane subunits are formed by the proteins As-48H and As-48E, and the protein As-48F plays an accessory role (Diaz et al., 2003). van Belkum & Vederas (2012) showed that the full production of carnocyclin A could only be achieved when a transformant carrying cclBITCDA was complemented with a second plasmid containing cclEFGH. Additionally, this transformant showed a slight decrease in sensitivity to carnocyclin A, indicating that CclEFGH plays a role in immunity. The present work on AclA further confirms the importance of these ABC transporters in the secretion of cyclic bacteriocins.

The growth reduction observed for strains MB558 and MB576 may be due to a reduction in the immunity level of both strains, making them more sensitive to aureocyclin 4185. Although we could not detect bacteriocin production when the strains were grown in broth, the concentration of aureocyclin 4185 in the culture supernatant under the conditions of the experiments performed might be sufficient to affect the growth of mutants MB558 and MB576. Note that strain MB558 was less affected than strain MB576 (Fig. 3). This result is in agreement with the literature, as AclF should have only an accessory function in immunity.

Interestingly, mutant MB495 (aclX::Tn917-lac) was the most affected in growth, showing the most dramatic OD reduction (Fig. 3). Once mutant MB495 was a weak AclA-producer and its growth was largely affected, these results strongly support the assumption that AclX plays a role in both AclA production and immunity. Alternatively, the growth reduction observed for the mutants affected in functions that seem to be involved in bacteriocin externalization might be attributed to accumulation of aureocyclin 4185 inside the mutant cells.

The aclC gene, like As-48C, encodes a protein belonging to a family of membrane proteins containing the domain DUF95. Therefore, they probably are functional analogues. As-48C together with As-48D constitutes the ABC transporter for enterocin AS-48 and provides low levels of immunity (Martínez-Bueno et al., 1998). A similar role is proposed for AclC and AclD.

**Transcription analyses of aclA, orfA and aclB**

The expression of aclA, aclX and of a region comprising part of aclX to part of aclB (called aclX–aclB region) was investigated by RT-PCR experiments. As expected, an amplicon of 195 bp, corresponding to an mRNA containing the aclA gene, was detected in strain 4185, but not in its pRJ101-cured derivative, MB493 (Fig. 4, lanes 2 and 6, respectively). Interestingly, it was also possible to detect an amplicon with the same size in strain MB495 (Fig. 4, lane 4), which was considered a non-bacteriocin producer on solid medium (Table 2).

Investigation of aclX expression revealed an amplicon of 568 bp in strain 4185 (Fig. 5a, lane 2), confirming the transcription of this gene and the presence of a functional

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**Fig. 3.** Kinetics of growth of the wild-type strain 4185 and the pRJ101::Tn917-lac mutants MB495, MB558, MB576 and MB594 at 37 °C. The results are the mean of eight readings performed in two independent experiments (done in quadruplicate). Blank, culture medium without bacterium.
promoter upstream of aclX. However, when strain MB495 (aclX::Tn917-lac) was analysed, no amplicon could be detected (Fig. 5b, lane 2), indicating that aclX is not expressed in this mutant.

Finally, expression analysis of the aclX–aclB region revealed an amplicon of 756 bp in both strains 4185 (Fig. 5c, lane 3) and MB495 (Fig. 5d, lane 3), suggesting that aclX and aclB may be transcribed as an operon, once no putative transcription terminator could be found downstream of aclX. By contrast, the results obtained with strain MB495 strongly support the assumption that aclB expression may also occur from a promoter located within the aclX sequence, as the Tn917-lac insertion abolished aclX expression in this mutant. In fact, a putative σ^A-dependent promoter (TTcAA – 16 nt – TATtAT) was located 238 bp downstream of the beginning of aclX (Fig. 1). This promoter may be involved in transcription of the transcript detected.

Together, these results show that the aureocyclicin 4185 structural gene aclA is expressed both in the producer strain 4185 and in the non-producer mutant MB495. By contrast, aclX is only expressed in strain 4185. Additionally, the Tn917-lac insertion upstream of aclX does not prevent aclB expression. It can therefore be concluded that aclX is part of the AclA gene cluster encoding a function necessary for bacteriocin production.

CONCLUSIONS

Many bacteria, including staphylococci, produce bacteriocins, and in several cases a single strain can produce multiple antimicrobial peptides (Heng et al., 2007). The production of multiple bacteriocins seems to be advantageous as it increases the fitness level and survival of a micro-organism in a competitive environment (Martin-Visscher et al., 2008). Staphylococcus aureus 4185, a strain involved in bovine mastitis, produces at least three antimicrobial peptides. Based on the present study, one such bacteriocin, aureocyclicin 4185, is a novel cyclic peptide that shares several similarities with other circular bacteriocins. Aureocyclicin 4185 is predicted to be cationic, to have a high content of hydrophobic residues and to belong to group I of the cyclic bacteriocins (van Belkum et al., 2011). Its gene cluster (aclX, aclB, aclI, aclT, aclC, aclD, aclA, aclF, aclG and aclH) was quite similar, but not identical, to the organization of the gene cluster responsible for carnocyclin A production and immunity. The carnocyclin A gene cluster has a gene, cclE, absent from the aureocyclicin 4185 gene cluster. The aureocyclicin 4185 gene cluster has a gene, aclX, not found thus far in the gene clusters of other cyclic bacteriocins. As CclE and AclX are both membrane-spanning proteins, AclX may be a functional homologue of CclE in strain 4185, contributing not only to aureocyclicin 4185 production but also to immunity to this novel cyclic bacteriocin.

Aureocyclicin 4185 could not be recovered from the culture supernatant of strains 4185 and MB608. Consistent with this finding was the absence of a peptide with the predicted
M, of AcLA (5607 Da) in the different HPLC fractions with antimicrobial activity, which were subjected to MS analyses in a previous study (Ceotto et al., 2010). These results suggest that aureocyclicin 4185 may be produced at very low levels. Alternatively, as described for many bacteriocins (Heng et al., 2007), the production of aureocyclicin 4185 might be under regulation, although no gene involved in bacteriocin regulation was found in its gene cluster. However, the regulatory genes might be chromosomally encoded. Attempts to get AcLA in the culture supernatant and to purify it for further characterization are currently in progress.

ACKNOWLEDGEMENTS

This study was supported by grants from CNPq, FAPERJ and CAPES to M. C. F. B.

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Edited by: M. Holden