Identification and Characterization of Two Novel Clostridial Bacteriocins, Circularin A and Closticin 574

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Received 28 June 2002/Accepted 1 November 2002

Two novel antibacterial peptides of clostridial species were purified, N-terminally sequenced, and characterized. Moreover, their structural genes were identified. Closticin 574 is an 82-amino-acid bacteriocin produced by Clostridium tyrobutyricum ADRIAT 932. The supernatant of the producing strain showed a high level of activity against the indicator strain C. tyrobutyricum. The protein is synthesized as a prepropeptide that is possibly secreted via the general secretion pathway, after which it is hydrolyzed at an Asp-Pro site. Circularin A is produced by Clostridium beijerinckii ATCC 25752 as a prepeptide of 72 amino acids. Cleavage of the prepeptide between the third leucine and fourth valine residues followed by a head-to-tail ligation between the N and C termini creates a circular antimicrobial peptide of 69 amino acids. The unusually small circularin A leader peptide of three amino acids is cleaved off in this process. The supernatant of C. beijerinckii ATCC 25752 showed a broad antibacterial activity range.

Clostridia have a negative image as many species are known as pathogens (50), toxin producers (50), and food-spoilage bacteria (36). Various clostridia produce bacteriocins, but despite their long history as a means of typing clostridia (33, 39), only little sequence, functional, or structural information is available on these antibacterial peptides. The few clostridial bacteriocins that have been further characterized are BCN5 and boticin B (12, 18). BCN5 is a large (97-kDa) UV-inducible bacteriocin. Moreover, their structural genes were identified. Closticin 574 is produced by Clostridium tyrobutyricum ADRIAT 932. The supernatant of the producing strain showed a high level of activity against the indicator strain C. tyrobutyricum. The protein is synthesized as a prepropeptide that is possibly secreted via the general secretion pathway, after which it is hydrolyzed at an Asp-Pro site. Circularin A is produced by Clostridium beijerinckii ATCC 25752 as a prepeptide of 72 amino acids. Cleavage of the prepeptide between the third leucine and fourth valine residues followed by a head-to-tail ligation between the N and C termini creates a circular antimicrobial peptide of 69 amino acids. The unusually small circularin A leader peptide of three amino acids is cleaved off in this process. The supernatant of C. beijerinckii ATCC 25752 showed a broad antibacterial activity range.

Closticin 574 is produced by Clostridium tyrobutyricum ADRIAT 932 and belongs to the class II bacteriocins. Circularin A, produced by Clostridium beijerinckii ATCC 25752, is shown to

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be a cyclic antibacterial peptide. The bacteriocins were purified from culture supernatants of the respective producer strains, and then the structural genes were cloned via reverse genetics techniques.

**MATERIALS AND METHODS**

**Bacterial strains, media, and reagents.** The strains and plasmids used in this study are listed in Table 1. Clostridia were grown anaerobically at 30°C in reinforced Clostridium medium (RCM) (Merck, Darmstadt, Germany) or in AC broth (Difco, Detroit, Mich.) for bacteriocin production. Cultures were grown anaerobically by flushing with oxygen-depleted nitrogen or by chemical absorption of the oxygen. Medium was heated for 5 min at 120°C to evacuate gases and chilled on ice. After inoculation, a cotton cloth with an equal volume of 10% pyrogallol (MERCK-Schuchardt, Hohenbrunn, Germany) and saturated sodium carbonate was applied and the tube was sealed airtight. Plates were incubated in anaerobic jars with an anaerocult A sachet (Merck). Lactobacilli and pediococci were grown in MRS at 30 and 37°C, respectively.

**Screening for bacteriocin production by clostridia.** Bacteriocin production was determined by colony overlay assays performed according to the protocol of Wray et al. (62).

**Bacteriocin assays.** Colony overlay assays were performed. Colonies on a nutrient plate were treated with chloroform vapor for 15 min. After 30 min of exposure to air, the colonies were covered with a top agar (0.75%) layer over the proper growth medium seeded with the indicator strain. After appropriate incubation, 1 to 3 days depending on the indicator strain used, the plates were examined for halos of growth inhibition. Bacteriocin activity was determined by a critical dilution assay as described by Geis et al. (20), except that assays were performed in microtiter plates. To 50 μl of serial-diluted bacteriocin-containing samples, 150 μl of medium containing the indicator strain Lactobacillus sake ATCC 15521 (pMG36e, pMG36c) was added, unless mentioned otherwise. The volumes were 100 μl for both sample and indicator when clostridia were used as indicators. After the appropriate incubation time (16 to 24 h for L. sake or 3 days for the microtiter plates), the microtiter plates were examined for growth inhibition. Inhibition is seen when the optical density at 600 nm in a well is less then half of that of a control well without added bacteriocin. The highest dilution of bacteriocin still inhibiting growth of the indicator is set to contain 1 arbitrary unit (AU). Bacteriocin production during growth was determined by growing the producer anaerobically in AC medium in krim seal bottles in a nitrogen atmosphere. Samples were taken by syringe at regular time points without disturbing the anaerobicity. To determine the bacteriocin activity in a Tricine–SDS-polyacrylamide gel, the procedure of Bhunia et al. (7) was used with the following modifications. Fixation of the gel was done by incubation in 20% isopropanol–10% acetic acid in water for 30 min at room temperature. Subsequently, the gel was washed six times with demineralized water, covered with a top agar (0.75%) layer containing 10−2 diluted cells of an overnight culture of the indicator L. sake, and incubated overnight at 30°C.

**Bacteriocin characterization.** Screening for bacteriocin production by clostridia was performed by measuring the antibacterial activity of filter-sterilized supernatants of stationary-phase cultures grown in RCM basically as described previously (20), except that the experiments were performed in 200-μl final volumes in microtiter plate wells. Microtiter plates were incubated anaerobically for 3 days before inhibition levels were determined. Additionally, bacteriocin production on plates was tested by a colony overlay assay. Six clostralid strains were used as indicators. The proteinase sensitivity of putative bacteriocin activities was determined by incubating filter-sterilized supernatants of stationary-phase cultures of bacteriocin-producing strains for 1 h (circularin 574) or 24 h (circularin A) at 37°C with an equal volume of 1.0 mg of proteinase K (Merck), thermolysin, alpha-chymotrypsin (Sigma, St. Louis, Mo.), or pronase (Roche) per ml or 0.78 mg of trypsin (Sigma/ml) in 0.1 M Tris buffer (pH 7.6). The residual bacteriocin activity was determined in a critical dilution assay. Bacteriocin temperature stability was determined by incubating filter-sterilized supernatants for 1 h at temperatures ranging from 4 to 90°C. After incubation, samples were cooled on ice and residual activity was measured in a critical dilution assay. Antibacterial activity as a result of the presence of a phase in the culture supernatant was excluded by filtering the culture supernatants through a 0.2-μm-pore-size filter (Schleicher & Schuell, Dassel, Germany) and testing the filtrate for antibacterial activity. The requirement of disulfide bridges for bacteriocin activity was determined by measuring the activity in a critical dilution assay in the presence of 10 mM dithiothreitol (DTT). The bacteriocin activity range was determined in a microtiter plate assay. Two hundred microliters of an exponentially growing culture of an indicator strain, diluted either 1:500 or 1:1,000 in fresh medium, was added to 50 μl of bacteriocin-containing supernatant. Plates were incubated at the appropriate temperature for 18 h, and growth was determined by measuring the optical cell density at 600 nm in a microtiter plate reader (Tecan, Männedorf, Switzerland).

**Bacteriocin purification.** For the purification of circularin A, Clostridium acetobutylicum ATCC 25752 was grown on an AC medium dialysate. The dialysate was obtained by dialyzing 100 ml of 10% concentrated medium against 900 ml of water by using a dialysis membrane (Medicell, London, England) with a cutoff of 12 to 14 kDa.

The supernatant of a culture of a clostridial strain, diluted either 1:500 or 1:1,000 in fresh medium, was added to 50 μl of bacteriocin-containing supernatant. Plates were incubated at the appropriate temperature for 18 h, and growth was determined by measuring the optical cell density at 600 nm in a microtiter plate reader (Tecan, Männedorf, Switzerland). The bacteriocin activity range was determined in a microtiter plate assay. Two hundred microliters of an exponentially growing culture of an indicator strain, diluted either 1:500 or 1:1,000 in fresh medium, was added to 50 μl of bacteriocin-containing supernatant. Plates were incubated at the appropriate temperature for 18 h, and growth was determined by measuring the optical cell density at 600 nm in a microtiter plate reader (Tecan, Männedorf, Switzerland).

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The supernatant of a filter-sterilized culture was precipitated with 50% ammonium sulfate at 4°C for at least 4 h. The precipitate was spun down at 12,000 × g for 30 min at 4°C and dissolved in 30 ml of demineralized water. This solution was extracted with an equal volume of n-butanol. The n-butanol phase was freeze-dried, and the resulting pellet was further purified by high-performance liquid chromatography (HPLC) with a C₄ column and eluted with a 10 to 90% acetonitrile gradient in a 0.1 to 0.05% trifluoroacetic acid gradient at 1 ml/min while the absorbance at 214 nm was

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**TABLE 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. beijerinckii ATCC 25752</td>
<td>Circularin A producer</td>
<td>NIZO</td>
</tr>
<tr>
<td>C. tyrobutyricum NIZO B570</td>
<td>Closticin 574 producer</td>
<td>NIZO</td>
</tr>
<tr>
<td>C. tyrobutyricum ADRIAT 932</td>
<td>Closticin 574 producer</td>
<td>NIZO</td>
</tr>
<tr>
<td>C. tyrobutyricum NIZO B575</td>
<td>Lactobacillus brevis L4</td>
<td>Unilever</td>
</tr>
<tr>
<td>C. tyrobutyricum CNRZ580</td>
<td>Lactobacillus buchneri L4</td>
<td>Unilever</td>
</tr>
<tr>
<td>C. tyrobutyricum NIZO B590</td>
<td>Lactobacillus casei subsp. casei L37</td>
<td>Unilever</td>
</tr>
<tr>
<td>Lactobacillus plantarum ATCC 14917</td>
<td>Lab collection</td>
<td>Unilever</td>
</tr>
<tr>
<td>L. sake ATCC 15521</td>
<td>Lab collection</td>
<td>Unilever</td>
</tr>
<tr>
<td>L. sake IFO12456</td>
<td>Lab collection</td>
<td>Unilever</td>
</tr>
<tr>
<td>L. lactis IL1403</td>
<td>Plasmid free</td>
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</tr>
<tr>
<td>L. lactis MG1363</td>
<td>Plasmid free</td>
<td>19</td>
</tr>
<tr>
<td>Pediococcus pentosaceus FBB61-2</td>
<td>Lab collection</td>
<td></td>
</tr>
<tr>
<td>Pediococcus pentosaceus PPE</td>
<td>1.2</td>
<td>49</td>
</tr>
</tbody>
</table>

* NIZO, NIZO food research (Ede, The Netherlands). Unilever is located in Vlaardingen, The Netherlands.
RESULTS

Bacteriocin production by clostridia. Twelve clostridial strains were tested for their ability to inhibit C. tyrobutyricum ADRIAT 932 and C. tyrobutyricum ADRIAT 932 exhibited antibacterial activity towards the indicator strain. The antibacterial activities produced by the C. beijerinckii strain and the C. tyrobutyricum strain were named circularin A and closticin 574, respectively, based on the results presented below and were further characterized. Production of the antibacterial activities was shown to take place in the exponential growth phase of each strain although circularin 574 production seems to be completed before the culture enters the stationary phase. (Fig. 1). The treatment of culture supernatants with proteinases caused complete loss of circularin 574 activity while circularin A activity was more resistant to proteinase digestion. Circularin A activity was only completely lost when the supernatant of producing cells was treated with proteinase K (1.0 mg/ml), whereas treatment with other proteinases such as thermolysin (1.0 mg/ml), pronase (1.0 mg/ml), trypsin (0.78 mg/ml), and alpha-chymotrypsin (1.0 mg/ml) resulted in residual antibacterial activity (2 to 100%) even after 24 h of incubation. Samples of circularin A-containing supernatant after proteinase digestion were analyzed by a gel overlay assay. The band of inhibition of growth of the indicator had not shifted, indicating that digestion products of circularin A are not the cause of the residual antibacterial activity (data not shown). These results are indicative of the proteinaceous nature of both antibacterial activities. Incubation at 80 or 90°C for 1 h causes complete inactivation of both bacteriocins. Incubation at somewhat less-elevated temperatures (between 50 and 80°C) causes a significant reduction in circularin 574 activity while circularin A activity is stable at these temperatures. The
bacteriocins do not seem to be extremely oxygen sensitive, as both inhibit *L. sake* ATCC 15521 in the presence of oxygen. All of these properties define both antibacterial activities as bacteriocins. DTT did not destroy the activity of either bacteriocin (data not shown), indicating that both probably do not contain the disulfide bridges necessary for antibacterial activity.

The activity spectrum of both bacteriocins was determined (Table 2). Circularin A has the widest activity range, inhibiting all *C. tyrobutyricum* strains tested and also lactococci, enterococci, and some *Lactobacillus* strains. Closticin 574 also inhibits all *C. tyrobutyricum* strains tested, except for the producer strain, and some lactobacilli but not the enterococci and *Lactococcus* tested. Circularin A and closticin 574 are both small proteins. The supernatant of *C. beijerinckii* ATCC 25752 produced a band of inhibition at a position of a protein of 2.2 kDa in a gel overlay assay (Fig. 2). The supernatant of the closticin 574 producer gave a growth-inhibition halo at the position of a protein of approximately 7 kDa (Fig. 2). When 2× Laemmli sample buffer with SDS and β-mercaptoethanol was used for sample preparation, no activity of closticin 574 was observed in the gel overlay assay. Omitting SDS and β-mercaptoethanol solved this problem. Circularin A was not affected by these constituents of the sample buffer. These results indicate that closticin 574, unlike circularin A, is sensitive to denaturing conditions.

**Circularin A purification.** The initial purification of circularin A was performed by using nondialyzed complete AC medium. However, impurity of the bacteriocin preparation with medium peptides precluded proper determination of the N-terminal amino acid sequence of circularin A. The use of dialyzed medium (see Materials and Methods) solved this problem. Production in dialyzed medium was between 8- to 38-fold lower than production in complete medium. The total bacteriocin activity in a stationary-phase culture grown in dialyzed medium was approximately 6.4 × 10^2 AU/mL. Using 50% ammonium sulfate, 31% of the activity could be precipitated. The precipitate was dissolved in demineralized water and extracted with *n*-butanol. No antibacterial activity was lost during this step of the isolation procedure. The *n*-butanol fraction was lyophilized, the residue was dissolved in water, and the sample was subjected to HPLC purification. Bacteriocin activity eluted in two peaks of which the first had the highest antibacterial activity (50 to 70% of the total activity applied). The second peak had less than 1% of the antibacterial activity of the first peak. Whether this peak is related to circularin A or is caused by another bacteriocin is unknown. Because of the low level of bacteriocin activity it was not further studied. The purified protein in the first peak was judged pure by Tricine–SDS-PAGE and silver staining, and the amount was estimated to be approximately 100 pmol (data not shown).

**Internal amino acid sequence of circularin A and identification of the cirA structural gene by reverse genetics.** Initial attempts to determine the N-terminal amino acid sequence of purified circularin A failed, indicating that the N terminus may be blocked. After cyanogen bromide (CnBr) cleavage of the protein, a mixture of two signals was obtained during N-terminal sequencing. This result indicated that the protein was cut into two fragments and contained at least two methionine residues. The mixture of fragments (A and B) after CnBr cleavage was purified by HPLC-C18, and the N-terminal amino acid sequence of fragment A was determined (Fig. 3). Combining the amino acid sequence of this fragment with the N-terminal amino acid sequence determined on the fragment mixture obtained after CnBr cleavage enabled deduction of the amino acid sequence of fragment B (Fig. 3).

In *C. beijerinckii* ATCC 25752, no plasmids could be detected (data not shown), and therefore, the genes for bacteriocin production are chromosomally located. Based on the amino acid sequences of the two fragments A and B, a number of degenerate primers were designed and used in different combinations as the relative orientation of fragments A and B was a priori unknown. A 75-bp PCR fragment was obtained from chromosomal DNA from *C. beijerinckii* ATCC 25752 with primers 514CB1a and 514CB2a. This result indicated that the fragments A and B occur in the order A to B in the bacteriocin (Fig. 3). The PCR fragment was subcloned in

### Table 2. Activity spectrum of circularin A and closticin 574

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>Circularin A</th>
<th>Closticin 574</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. beijerinckii</em> ATCC 25752</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><em>C. tyrobutyricum</em> NIZO B570</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. tyrobutyricum</em> ADRIAT 932</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>C. tyrobutyricum</em> NIZO B590</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. tyrobutyricum</em> CNRZ500</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>E. faecalis</em> JH1-2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>E. faecalis</em> OG1X</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus</em> alimentarius L4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus</em> brevis L40</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus</em> buchneri L4</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus</em> casei subsp. casei L37</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Lactobacillus</em> plantarum lacC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Lactobacillus</em> sake ATCC 15521</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>–</td>
</tr>
<tr>
<td><em>L. lactis</em> IL1403</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>L. lactis</em> MG1363</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Pediococcus</em> pentosaceus FBB61-2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>Pediococcus</em> pentosaceus PPE 1.2</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*+, inhibition; –, no inhibition of the indicator strain by 5×-diluted culture supernatant of *C. beijerinckii* ATCC 25752 (circularin A producer) or *C. tyrobutyricum* ADRIAT 932 (closticin 574 producer) in a microtiter plate assay.*
pUC19 and sequenced. The entire structural gene of circularin A was subsequently cloned by inverse PCR techniques and sequenced (Fig. 3). The sequence is available under GenBank accession no. AY164463.

CirA codes for a peptide of 72 amino acid residues. The gene is preceded by a proper Shine-Dalgarno sequence (AAGGAGGT) at a distance of 6 nucleotides upstream of the translation initiation codon. Putative −35 (TT GCGA) and −10 (TATCAA) sequences are present. A large palindromic structure (ΔG₀ = −20.7 kcal) is present downstream of cirA. Performing a BLAST search with the derived
Amino acid sequence of circularin A revealed that the protein has 60% similarity (30% identity) with the E. faecalis bacteriocin AS-48 (40). AS-48 is a circular molecule, and the purified peptide was also blocked at the N terminus (40). Continuing the N-terminal amino acid sequence reactions on the CnBr-cleaved fragment mixture, the sequence of fragment B was extended. In this way it was shown that circularin A is also a circular peptide. It also allowed determination of the exact point of circularization (Fig. 3). Mature circularin A contains 69 amino acids. It is formed by the removal of a leader of three amino acid residues from the preprotein and head-to-tail ligation between the processed N terminus and the C terminus. Circularin A has high pi of 10.60 as calculated on the noncircular form of the mature bacteriocin, which is common for bacteriocins.

Purification of closticin 574. The supernatant of a producing culture of C. tyrobutyricum ADRIAT 932 containing 6.4 × 10^7 AU of bacteriocin activity was precipitated with 40% ammonium sulfate. In this way, 80% of the total activity was recovered. The precipitate, dissolved in 20 ml of 50 mM phosphate buffer (pH 7.0) with 30% isopropanol and 1.0 mM ammonium sulfate, was purified by FPLC with an ammonium sulfate gradient (see Materials and Methods). Bacteriocin activity eluted around 300 mM ammonium sulfate with 30% isopropanol, and the recovery of activity after the FPLC step was 100%. The pooled active fractions were concentrated by ultrafiltration and DEAE-column chromatography applying a NaCl gradient. Of the total activity, 35% was present in the flowthrough while 18% of the activity eluted between 1.6 and 2.0 M NaCl. The loss of bacteriocin activity could be caused by degradation and/or chemical modification of the bacteriocin or could indicate that the total bacteriocin activity is based on more than one protein. The flowthrough was concentrated by column ultrafiltration and subjected to HPLC. The activity retrieved after this step was only about 20% of that applied to the column. The quantity (10 to 20 pmol) and purity were sufficient, as judged by Tricine–SDS-PAGE and silver staining, to allow for N-terminal amino acid sequence determination by Edman degradation.

FIG. 4. Nucleotide and derived amino acid sequences of closticin 574. (A) N-terminal amino acid sequence of closticin 574 as determined by Edman degradation on the purified protein. Amino acids within parentheses were determined with a confidence level of >90% certainty. Parenthetic amino acids with a question mark obtained a confidence level of 50%. At position 16, signals of both Ala and Gly were derived, probably as a consequence of high background levels of glycine. (B) Nucleotide and deduced amino acid sequences of the structural gene of closticin 574 (cloA). The deduced amino acid sequence of mature closticin 574 is indicated in boldface. The predicted N-terminal signal sequence is underlined. Putative Shine Dalgarno (S.D) and promoter sequences are underlined. The putative UUG start codon is indicated in boldface.
N-terminal amino acid sequence of closticin 574 and isolation of the cloA structural gene. The 27 N-terminal amino acid residues of purified closticin 574 were determined (Fig. 4). Degenerate primers B574-for4 and B574-rev1 were designed based on this sequence and used for inverse PCR on Sau3A-digested and self-ligated *C. tyrobutyricum* ADRIAT 932 chromosomal DNA. A 265-bp PCR fragment was obtained, cloned in the pCR-Blunt II-TOPO vector, and sequenced. Based on the obtained sequence, primers were designed and used for further PCR and sequencing. The nucleotide sequence of the structural gene of closticin 574 (cloA) is presented in Fig. 4. The sequence is available under GenBank accession no. AY164462. From the nucleotide and N-terminal amino acid sequence data, closticin 574 is concluded to be initially produced as a preproprotein of 310 amino acid residues. It is subsequently processed to a bacteriocin of 82 amino acids with a pI of 9.9. The protein has an unusually long preproprotein sequence of 228 amino acids. Translation initiation putatively starts at a rare UUG start codon. A putative ribosome binding site (GGAG) is present 5 nucleotides upstream of this start codon, and the cloA gene is preceded by putative −35 (CTG AAA) and −10 (GATAAT) sequences. The deduced amino acid sequence of cloA is almost identical to the sequence obtained by Edman degradation. Arg9 and Arg15 in the Edman degradation profile are actually cysteine and tryptophan residues, respectively, in the deduced peptide. CloA is homologous to the deduced products of two unknown open reading frames of *Corynebacterium diphtheriae* (http://www.sanger.ac.uk) and to one unknown product of *Corynebacterium glutamicum* (accession no. AX127147). The first 27 amino acids of the primary translation product of cloA were identified as a signal sequence by the SignalP program (Fig. 4).

**DISCUSSION**

Closticin 574 is, on the basis of the nucleotide sequence of its structural gene, produced as preproprotein of 309 amino acid residues. After secretion and processing, it gives rise to an antimicrobial peptide of 82 amino acid residues. The first 27 amino acids of the full-length protein probably constitute a general signal peptide, as predicted by the SignalP program. General signal peptides are uncommon for bacteriocins, although some bacteriocins, such as enterocin P, diversicin A, and lysteriocin (9, 30, 61), are secreted via the general secretion pathway. Furthermore, secretion of a bacteriocin that normally is externalized by dedicated, sec-independent secretion systems can be accomplished by fusing it to a signal peptide directing the heterologous protein to the general secretion pathway (5, 42). The predicted secretion of CloA via the sec pathway and subsequent processing of the putative signal peptide would not yield the bioactive peptide. The secreted protein is most probably further processed extracellularly either by a general or by a specific protease. Such a mechanism of bacteriocin activation has been observed before. The bacteriocin Lsb1 of *Lactococcus lactis*, for instance, has been recently shown to be processed, and thus activated, after secretion by the cell wall protease HtrA (16a). It is also possible that the aspartyl-prolyl bond (Asp<sup>227</sup>-Pro<sup>228</sup>) at which cleavage in closticin 574 has taken place is chemically broken to create the active peptide, as is the case for guanylin (54). However, despite the fact that the N-terminally sequenced protein was active, we cannot exclude the possibility that the cleavage of the Asp<sup>227</sup>-Pro<sup>228</sup> bond is the result of the purification method, as Asp-Pro bonds are easily hydrolyzed under acidic conditions (38, 48). During purification of closticin 574, such acidic conditions are encountered in the HPLC step when the sample is eluted in the presence of trifluoroacetic acid, a very acidic compound. Cleavage of Asp-Pro bonds during protein purification has been observed previously (16, 54, 55, 57).

The cysteines present in closticin 574 are not involved in the formation of a disulfide bridge required for activity since DTT treatment did not inactivate the protein. With all data taken together, closticin A classifies as a class Ic bacteriocin, although it is exceptional for its unusual maturation process. The classification is based on its sensitivity for proteinases and the relatively small size (82 amino acid residues) (35) of the processed prepropeptide. Closticin A is not structurally modified like the class I bacteriocins (51), since none of the characteristic residues and problems typical when sequencing proteins contain lanthionine, β-methylanthionine, or dehydroalanine residues (51) were encountered during N-terminal sequencing. The bacteriocin does not contain the YNGNVXAAAC consensus motif of the pediocin-like bacteriocins (15), nor are two different peptides required for activity, as is the case for lactococcin G, lactococcin M/N, and plantaricins EF and JK (3, 47, 61).

Circularin A is produced as a preproprotein of 72 amino acids that is processed to a circular peptide of 69 amino acid residues. The preprotein is circularized by the hydrolysis of the peptide bond between Leu<sup>1</sup> and Val<sup>4</sup> and the formation of a new bond between Val<sup>4</sup> and the C-terminal Tyr<sup>72</sup> (Fig. 3). This head-to-tail linkage has so far only been described for three other proteins, gassericin A, microcin J25, and AS-48, all of which are antibacterial peptides (6, 31, 40). The circularization of microcin J25 is accomplished by two dedicated proteins (36).

For AS-48, it is not known which proteins, out of four different proteins involved in the production of the active bacteriocin, are responsible for the actual circularization (41). The actual location at which circularization takes place is not known for any of the four bacteriocins. The homology between the two systems for production of microcin J25 or AS-48 is limited to the presence of an ATP-binding site in one of the proteins involved in each case. Circularin A is similar to AS-48 but, interestingly, lacks the extended leader of AS-48 (40). Only the first three N-terminal amino acids of pre-CirA are cleaved off to allow the formation of a peptide bond between Val<sup>4</sup> and Tyr<sup>72</sup>. The three other circular bacteriocins are translated with longer leader peptides of 33, 35, and 37 residues for gassericin A, AS-48, and microcin J25, respectively (6, 31, 40). The question arises as to whether the 3-amino-acid-long leader in pre-CirA has a function in targeting the protein for secretion or processing, as do the signal peptides and the double glycine leaders of other bacteriocins (23, 60). Some bacteriocins are known to be secreted without the need for a leader peptide (9, 16a, 45), but these bacteriocins are not modified nor is the transport machinery dedicated to that bacteriocin. It has been postulated that in those cases the overall charge is the recognizing factor (16a). Another possibility might be that the signal directing the protein for modification and secretion is embedded within the peptide, as has been shown for hemolysin (34),...
some metalloproteases (21), and some peroxisomal proteins (58). That a few amino acid residues can already have a targeting function has been shown for the peroxisome-targeting signals PTS1 and PTS2. PTS1 is a conserved C-terminal tripeptide sequence while PTS2 is a nonapeptide sequence located near the N terminus or at internal locations of the protein to be targeted (58). If the 3-amino-acid residue leader peptide of pre-circularin A embeds a targeting function, it would be the smallest leader peptide known.

Circularin A is highly resistant to digestion by sequence-specific endoproteinasises. This is not due to absence of digestion sites, since sites for trypsin (Lys-X, Arg-X), alpha-chymotrypsin (FYW-X [not P,M]), and thermolysin (X [not D,E]-A, F, I, L, M, V) are present in circularin A. This same resistance to proteolytic digestion has been observed for microcin J25 (6). AS-48 was resistant to digestion with alpha-chymotrypsin (29) but was hydrolyzed by trypsin, pronase, and proteasine K (17). We presume that the resistance to proteolytic hydrolysis is due to the inaccessibility of the recognition sites. This notion seems to be supported by the three-dimensional structure of the circularin A homologue AS-48. AS-48 is a tightly folded globular peptide consisting of 5 α-helices, resembling the typical saponin fold as observed in NK-lysin, a porcine antibacterial peptide from NK and T cells (22). The functionality of such resistance towards proteinasises could be to make the protein less susceptible to digestion by proteinasises of target bacteria, thereby increasing the activity range of the bacteriocin. For instance, the natural cell wall proteinases PrtPI and PrtPIII of \textit{L. lactis} digest bacteriocins such as LcnB, LenA, and Lsb2 (16a) but are unable to digest circularin A (unpublished data).

Classification of the circular bacteriocins circularin A, microcin J25, gasserin A, and AS-48 in either of the current bacteriocin classes I to III is not justifiable. They are ribosomally synthesized and posttranslationally modified small bacteriocins, which makes them fall out of bacteriocin classes II and III. Due to the fact that they do not contain modified amino acids, they do not belong to the class I bacteriocins, and since they are not complex proteins composed of a protein plus another chemical moiety (lipid or carbohydrate) needed for activity, they cannot be in class IV. We therefore propose to distinguish a new class, class V, of bacteriocins consisting of ribosomally synthesized nonmodified head-to-tail-ligated cyclic antibacterial peptides. Characteristic of class \( V \) bacteriocins is their cyclization by formation of a peptide bond between the \( N \) and \( C \) termini of a processed prepeptide. This process of leader peptide removal, circularization, and secretion putatively involves dedicated enzymatic reactions. These enzymatic reactions are most probably encoded by the genes in the direct vicinity of the structural gene (unpublished data).

ACKNOWLEDGMENTS

This work was supported by FCDF Corporate Research and the Dutch ministry of economic affairs.

We are grateful to M. J. Gasson (Institute of Food Research, Norwich Research Park, Colney, United Kingdom) for kindly providing the enterococcal strains used in this study. We thank Beata Ruban for skillful technical assistance.

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