Functional analysis of circular and linear bacteriocins of Gram-positive bacteria
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Functional Analysis of the Gene Cluster Involved in Circularin A, a Bacteriocin Produced by *Clostridium beijerinckii* ATCC 25752.

ABSTRACT

A region of 12 kb flanking the structural gene of the cyclic anti-bacterial peptide circularin A of Clostridium beijerinckii ATCC 25752 was sequenced and the putative proteins involved in the production and secretion of circularin A were identified. The genes are tightly organised in overlapping open reading frames. Heterologous expression of circularin A in Enterococcus faecalis was achieved and five genes were identified to be minimally required for bacteriocin production and secretion. Two of the putative proteins, CirB and CirC, are predicted to contain membrane spanning domains while CirD contains a highly conserved ATP-binding domain. Together with CirB and CirC this ATP-binding protein is involved in the production of circularin A. The fifth gene, cirE, confers immunity towards circularin A when expressed in either Lactococcus lactis or E. faecalis and is needed in order to allow the bacteria to produce bacteriocin. Additional resistance against circularin A is conferred by the activity of the putative transporter consisting of CirB and CirD.

INTRODUCTION

Antimicrobial peptides or bacteriocins are produced by various Gram-positive and Gram-negative bacteria. Although many bacteriocins inhibit the growth of strains closely related to the bacteriocin producer, an ever-increasing number of bacteriocins have a broader activity range. Only few anti-microbial peptides from clostridial origin have been characterized at the molecular level, despite their extensive use as a means of identifying and typing clostridia (24, 32). The three bacteriocins that have been partially characterized are BCN5, boticin B and circularin A (9, 12, 25) produced by Clostridium perfringens, C. botulinum and C. beijerinckii, respectively.

Circularin A, a circular bacteriocin produced by C. beijerinckii ATCC 25752, is active against a broad range of Gram-positive bacteria (25). The circularization of the peptide involves a head-to-tail peptide bond formation between the fourth and last amino acid of the precursor peptide (25). Circularin A shares limited sequence homology with enterocin AS-48 (also known as Bac21), a cyclic bacteriocin from Enterococcus faecalis (34, 56), but its precursor lacks the long leader present in the enterocin AS-48 precursor. The circularin A gene cluster is chromosomally located, while the enterocin AS-48 operon is located on a plasmid. Both circularin A and enterocin AS-48 belong to the recently defined Class V bacteriocins of ribosomally synthesized, non-modified, head-to-tail-ligated cyclic anti-bacterial peptides (25). Other Class V bacteriocins are microcin J25 and gassericin A (4, 22). Microcin J25, peptide
of 21 amino acid residues produced by *Escherichia coli*, is the only circular peptide known so far that is produced by a Gram-negative bacterium (4). The genes involved in the production of microcin J25 are located in an operon immediately downstream of the structural gene (51). Gassericin A is produced by *Lactobacillus gasseri* as a 91-amino-acid-precursor peptide that is circularized after removal of a leader peptide of 33 amino acids (22, 23). The coding regions of enterocin AS-48 of two strains have been sequenced and determined to be almost identical (35, 56). Enterocin AS-48 is a tightly packed peptide containing five α-helices and is structurally related to NK-lysin, a cytotoxic peptide from human natural killer or T-cells (16).

Most bacteriocins require processing of a precursor peptide in order to become (fully) active. For many bacteriocins the genes encoding processing, secretion and immunity functions flank the structural gene. Processing can involve modification of amino acids, as is the case in lantibiotics, leader peptide removal or, in the case of circular peptides, circularization. The mechanisms underlying these modifications are poorly understood although the proteins involved are generally known. The secretion of most bacteriocins occurs via dedicated ABC-transporters (26) while some can be secreted via the general secretion pathway (5, 21, 33).

Immunity systems for bacteriocins are poorly characterized, but it has been demonstrated that specialized immunity proteins confer immunity to cells from bacteriocin action by blocking access to a putative receptor, as is the case for the lactococcin A immunity protein LciA (58). In some cases ABC-transporters have been shown to be involved, e.g. the NisFEG system in nisin resistance and McbFE in microcin B17 resistance (13, 43). Little homology exists between bacteriocin immunity proteins, even between those that are involved in immunity against bacteriocins of the same class.

In this study we identify the genes required for functional heterologous expression of circularin A and we show that two independent mechanisms confer reduced circularin A sensitivity, one of which is based on the expression of *cirE* and the other on the combined expression of *cirB* and *cirD*. As such, it will further the field of clostridial bacteriocins and that of Class V (circular) bacteriocins in particular.

**MATERIAL AND METHODS**

**Bacterial strains, media and reagents.**
The strains and plasmids used in this study are listed in Table 1. *Clostridium beijerinckii* ATCC 25752 was grown anaerobically at 30°C in AC-broth (Difco, Detroit, MI, USA). Anaerobicity was obtained by chemical absorption of the oxygen in closed bottles as previously described (25). *Lactobacillus saké* ATCC 15521 was grown in De Man Rogosa and Sharpe (MRS; Merck, Darmstadt, Germany) broth at 30°C. Two-fold diluted M17 broth (Difco) with a final concentration of 1.9% β-glycerophosphate (Merck) and 0.5% glucose (G½M17)
was used for growth of *Lactococcus lactis* NZ9000 and *Enterococcus faecalis* JH2-2 at 30°C and 37°C, respectively. *Escherichia coli* DH5α was grown for 16 hours in Trypton-Yeast (TY) broth at 37°C with vigorous agitation (250 rpm). For growth on plates medium containing 1.5% agar was used. Ampicillin (Sigma, Zwijndrecht, the Netherlands) and chloramphenicol (Cm; Sigma) were used at 100 µg/ml and 10 µg/ml respectively for *E. coli*. Chloramphenicol and erythromycin (Em; Sigma) were used at 5 µg/ml each for *L. lactis* NZ9000 and at 20 µg/ml and 2 µg/ml, respectively, for *E. faecalis* JH2-2. When used together Cm and Em were employed at 2.5 µg/ml each for *Lactobacillus saké* ATCC 15521 or at 10 µg/ml Cm and 2 µg/ml Em for *E. faecalis* JH2-2.

Nucleotide sequencing.  
Inverse PCR techniques using the nucleotide sequence of cirA (25) were employed to obtain the region surrounding the cirA gene. PCR products were sequenced either directly or after subcloning of restriction enzyme digestion fragments in pUC19. PCR products were purified using the High Pure PCR Product Purification kit of Roche (Roche Diagnostics GmbH, Mannheim, Germany). Sequencing was performed using Cy5-labeled universal, reverse or T7 primers (Amersham Pharmacia Biotech Roosendaal, the Netherlands) and the ALFII-system (Amersham Pharmacia Biotech) according to the protocols of the supplier with the following modifications: the power was set at 15 W and at 18 W for the long-read and high-resolution gels, respectively.

Bacteriocin assays.  
Colony overlayer assays were performed as described previously (25). Bacteriocin activity in *C. beijerinckii* ATCC 25752 supernatant was quantified in triplicate by a critical dilution assay as described by Geis et al (14), with the modification that assays were performed in microtiterplates: to 50 µl of serially diluted, bacteriocin-containing samples 150 µl of medium containing the indicator strain *Lb. saké* ATCC 15521 (pMG36e; pMG36c), diluted 100-fold from a stationary phase overnight culture, were added, unless mentioned otherwise. Resistance to bacteriocin was determined by plating strains on plates containing 4 or 10% (v/v) filter-sterilized *C. beijerinckii* ATCC 25752 supernatant containing circularin A. Alternatively a critical dilution assay was performed using the strain of interest as an indicator strain. In these critical dilution assays 100 µl of bacteriocin-containing sample were mixed with 100 µl of freshly diluted (1000-fold) indicator strains.

Cloning methods and materials.  
Molecular cloning techniques were performed essentially as described by Sambrook et al. (45). Restriction enzymes, T4-DNA ligase and Expand DNA-polymerase were obtained from Roche (Roche Diagnostics GmbH) and used as specified by the supplier. *L. lactis* NZ9000 was transformed as described by Shepard and Gilmore (19) using 1% glycine (Merck). *E. faecalis* JH2-2 was transformed as described previously (47) [http://w3.ouhsc.edu/enterococcus] using 8% glycine. After transformation, both strains were plated on G/%M17 medium containing 0.5 M sucrose and the appropriate antibiotics. Plasmids from *L. lactis* NZ9000, *E. coli* DH5α and *E. faecalis* JH2-2 were isolated according to Birnboim (3), with the following modifications when using *E. faecalis*: mutanolysin (1 U/ml; Sigma) was added to the suspension buffer to facilitate lysis and plasmids isolated from 50 ml of culture were, after RNAase (0.5 mg/ml; Sigma) treatment, further purified using the High Pure PCR Product Purification kit (Roche Diagnostics GmbH).
### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. beijerinckii</em> ATCC 25752</td>
<td>Circularin A producer</td>
<td>NIZO</td>
</tr>
<tr>
<td><em>E. faecalis</em> JH2-2</td>
<td>plasmid free derivative of <em>E. faecalis</em> JH-2</td>
<td>(20)</td>
</tr>
<tr>
<td><em>L. lactis</em> NZ9000</td>
<td>plasmid free derivative of <em>L. lactis</em> MG1363, pepN::nisRK</td>
<td>(30)</td>
</tr>
<tr>
<td><em>Lb. saké</em> ATCC 15521</td>
<td>bacteriocin indicator</td>
<td>lab collection</td>
</tr>
<tr>
<td><em>E. coli</em> Top10</td>
<td></td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMG36c</td>
<td>Cm’, pWV01 based cloning vector carrying the strong lactococcal promoter P&lt;sub&gt;32&lt;/sub&gt;</td>
</tr>
<tr>
<td>pMG36e</td>
<td>Em’, gene expression vector carrying P&lt;sub&gt;32&lt;/sub&gt;</td>
</tr>
<tr>
<td>pCR21</td>
<td>Amp’, Km’</td>
</tr>
<tr>
<td>pCRAE</td>
<td>Amp’, Km’</td>
</tr>
<tr>
<td>pLL253</td>
<td>Em’, theta replicating cloning vector</td>
</tr>
<tr>
<td>pLL-E</td>
<td>Em’, pLL253 derivative with cirE under control of P&lt;sub&gt;32&lt;/sub&gt;</td>
</tr>
<tr>
<td>pLL-P32</td>
<td>Em’, pLL253 derivative with P&lt;sub&gt;32&lt;/sub&gt; promoter</td>
</tr>
<tr>
<td>pMGAE1</td>
<td>Cm’, pMG36c derivative with cirABCDE under control of P&lt;sub&gt;32&lt;/sub&gt;</td>
</tr>
<tr>
<td>pCir</td>
<td>Cm’, derivative of pMGAE1 overexpressing circularin A</td>
</tr>
<tr>
<td>pCirΔA</td>
<td>Cm’, pCir ΔcirA</td>
</tr>
<tr>
<td>pCirΔB</td>
<td>Cm’, pCir ΔcirB</td>
</tr>
<tr>
<td>pCirΔC</td>
<td>Cm’, pCir ΔcirC</td>
</tr>
<tr>
<td>pCirΔD</td>
<td>Cm’, pCir ΔcirD</td>
</tr>
<tr>
<td>pCirΔE</td>
<td>Cm’, pCir ΔcirE</td>
</tr>
<tr>
<td>pCirΔAE</td>
<td>Cm’, pCir ΔcirA, ΔcirE</td>
</tr>
<tr>
<td>pCirΔBE</td>
<td>Cm’, pCir ΔcirB, ΔcirE</td>
</tr>
<tr>
<td>pCirΔCE</td>
<td>Cm’, pCir ΔcirC, ΔcirE</td>
</tr>
<tr>
<td>pCirΔDE</td>
<td>Cm’, pCir ΔcirD, ΔcirE</td>
</tr>
<tr>
<td>pCirΔACE</td>
<td>Cm’, pCir ΔcirA, ΔcirC, ΔcirE</td>
</tr>
<tr>
<td>pCirΔABCE</td>
<td>Cm’, pCir ΔcirA, ΔcirB, ΔcirC, ΔcirE</td>
</tr>
<tr>
<td>pCirΔACDE</td>
<td>Cm’, pCir ΔcirA, ΔcirC, ΔcirD, ΔcirE</td>
</tr>
<tr>
<td>pMG-E</td>
<td>Em’, pMG36e derivative with cirE under control of P&lt;sub&gt;32&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

Amp’: ampicillin resistance Cm’: chloramphenicol resistance, Em’: erythromycin resistance, Km’: kanamycin resistance, * NIZO, NIZO food research (Ede, the Netherlands). Invitrogen (Breda, the Netherlands)
Cloning of the circularin A determinant.

The region encompassing cirA to cirE was amplified using primers located just upstream of cirA (B51426, 5'-ACCGTGCAGCTCATGAGTGTGTGTGTAAGGAGTGAATTAT GTTTTTATTGCAGG-3') and downstream of cirE (B51427, 5'-CGCGGATCCGTCGACCTCCACTTTAATGTTAGTTATGCTC-3'). SalI-RcaI and BamHI-SalI sites, respectively, in the two primers are underlined. All enzymes (Roche) were used according to the manufacturers instruction. The PCR product was cloned using the Zero-Blunt® TOPO® PCR cloning kit (Invitrogen, Breda, the Netherlands), creating pCRAE. The plasmid pCRAE was digested with SpeI and XhoI. The fragment carrying cirA was ligated into pMG36c digested with XbaI and SalI and the ligation mixture was used to transform E. coli DH5α. Transformants were identified by growth on TY-agar with chloramphenicol. The correct plasmid, pMGAE1, was isolated as described above and introduced into E. faecalis JH2-2. Three consecutive selection steps of clean streaking and testing for a strain with a high and stable bacteriocin expression phenotype using the colony overlay assay yielded E. faecalis JH2-2 carrying a pMGAE1 derivative labeled pCir.

In-frame deletions of cirA through cirD were made by amplifying pCir by PCR with appropriate outward facing primers, creating a PCR product of the entire plasmid but lacking the gene of interest. The primers used were 5'-AGTATGGCAAGAGCTATAGC-3', 5'-CACGCTAGTGCTCCTGC-3' for ∆cirA, 5'-TAATTATGCCGTATCACC-3', 5'-CCAAGAGTTATAGTTGAGT CG-3', for ∆cirB, 5'-GTGCACATAGTAGGATTITTAAG-3', 5'-GAAACATTC  AACAATAATACC-3' for ∆cirC and 5'- GAACCTATCTAGTAAAAGGAAG-3', 5'-AGTTATCTCTAGATAGGCTTC-3' for ∆cirD. Each PCR product was kinated using T4-poly nucleotide kinase (Amersham Pharmacia Biot ech) in T4-ligase buffer (Roche Diagnostics GmbH) and subsequently self-ligated using T4-ligase creating the plasmids pCir∆A, pCir∆B, pCir∆C and pCir∆D. Derivatives of pCir with a deletion of cirE and a deletion of one of the other genes cirABCD, pCir∆E, pCir∆AE or pCir∆DE, were made by the same method using the primers 5'-CATATATTCTACCTTTTCT-3', 5'-GTAATTAAAGGCTCTAATAAG-3' for ∆cirE and the plasmids carrying the respective single deletions in the PCR. A plasmid with a triple deletion, pCir∆ACE, was constructed likewise by deleting cirC using pCir∆AE as a template and the primers used for the single deletion of cirC. Based on pCir∆ACE, pCir∆ABCE and pCir∆ACDE were made using the primers employed for the single knockouts of cirB and cirD, respectively. All plasmids were isolated using E. faecalis JH2-2 as the cloning host.

The cirE gene was cloned behind the lactococcal chromosomal P32 promoter by digesting the PCR product obtained with primers B51426 and B51427 with HpaI and Sall and ligating the cirE carrying fragment into Smal and Sall digested pMG36e, leading to pMG-E. Colonies, obtained after transformation of L. lactis NZ9000, were replica streaked onto G2M17 plates with 4% (v/v) C. beijerinckii ATCC 252752 supernatant to screen for circularin A immunity. The plasmid was isolated and used to transform E. faecalis JH2-2. In order to make pCir∆E, a fragment with the immunity gene cirE behind the P32 promoter was first cloned in pIL253, to avoid possible lethal effects of bacteriocin expression without immunity. This was done by digesting pMG-E with EcoRI and Sall and ligating the cirE carrying fragment into pIL253 digested with the same enzymes. The resulting plasmid (pIL-E) was subsequently made using pCir as a template and the appropriate primers. E. faecalis JH2-2 (pIL-E) was used as the host for construction of pCir∆E.

Computational analyses.

Open reading frames were identified using the Glimmer 2.0 program (7). Predictions by the Glimmer 2.0 program were manually checked for validity. Homology comparisons were performed using the basic local alignment tool (BLAST) as described by Altschul et al. (1). BLAST searches were performed against the NCBI non-redundant protein database and the NCBI microbial genomes database.
Homologies with conserved domains from the Pfam database were also identified with BLAST searches. Putative signal peptides were identified using signalP (38). Putative transmembrane helices were identified using the TMHMM2.0 program (28). Dyad symmetries, isoelectric points and molecular weights were determined using the program Clonemanager 4 (SEcentral; Scientific & Educational software). Sequence alignments were performed with the ClustalW program available at (54).

RESULTS

Sequence analysis of the region encompassing the circularin A structural gene.

The structural gene of the circular bacteriocin circularin A of Clostridium beijerinckii ATCC 25752 (cirA) has previously been cloned and sequenced (25). A region of 11 kb surrounding cirA was sequenced and shown to contain twelve open reading frames (ORFs) including cirA (Figs. 1 and 2). The sequence is available under GenBank accession no AJ566621. Six putative promoters were identified, one upstream of each cfgR (CirA flanking gene response regulator), cfg01 (CirA flanking gene 01), cfg02, cirA, cirB and cirG. The cirBCDE and cirGHI genes are putatively transcribed as polycistronic messengers, since no clear transcription initiation signals were detected other than the ones upstream of cirB and cirG, respectively. Translation of cirC and cirD can putatively start from alternative start codons within the same reading frame (Fig. 2). Overlap between the end of one gene (cfgR, cirC, cirD, cirH and cirI) and the predicted start of the downstream gene, which is suggestive of regulation of expression by translational coupling, is a common feature in the entire region (Fig. 2). For cirH, translational coupling to cirG would be the only means of expression as it lacks an obvious ribosome binding site (Fig. 2).

Derived protein sequences and homologies are presented in Table 2. The gene products CirB through to CirI all show some degree of homology to proteins involved in the production of enterocin AS-48, a bacteriocin produced by Enterococcus faecalis S-48 (34). Homologues of the putative proteins AS-48C1 and Bac21F are not encoded by the cir operon. CirD and CirH, like AS-48D and BacH, each contain an ATP-binding domain (Fig. 3). CirG belongs to the HlyD family of accessory proteins of ABC-transporters, which includes proteins like EmrA, an accessory protein in the EmrAB multidrug transporter (31), and LcnD, an auxiliary protein involved in the secretion of the bacteriocin lactococcin A (11). Based on the occurrence of the G-X[9]-F-X[10]-G motif, CirI can be classified in the ortholog group 3-1 of ABC-transporters, as defined by Tomii and Kanehisa (55). Like the already characterized members of this group (FtsX (6), LoIC and LoIE (37)) CirI contains four putative transmembrane domains,
Figure 1: (A) Physical map of the region surrounding the circularin A structural gene cirA of C. beijerinckii ATCC 25752. Filled arrows indicate genes; bent arrows are putative promoters; lollypops represent predicted regions of dyad symmetry (ΔG<−10 kcal/mol); dotted arrows show possible polycistronic messengers. Map units is bp. (B) Schematic representation of the cir-DNA fragments in the indicated plasmids and location of the deletions (indicated by the thin lines). Open arrowheads indicate a deletion in cirE. Promoters are shown by bent arrows; CirA+: circularin A production; CirR+: circularin A resistance; + +, full protection against CirA (>24-fold increase relative to E. faecalis JH2-2 (pMG36c)); +, partial protection (2-16 fold increase relative to E. faecalis JH2-2 (pMG36c)); −, sensitive;

typical for group 3-1 ABC transporters (Fig. 4) and a Duf214 domain of predicted permeases as defined in the Pfam database. CfgR and CfgK are homologous to, respectively, response regulators and histidine kinases of two component regulatory systems. Cfg01 is homologous (24% identity) to the accessory gene regulator (AgrB) of Staphylococcus aureus, which is thought to be involved in processing and secretion of a signaling peptide (AgrD), thereby regulating a large set of virulence factors (61). Cfg02 is homologous to two putative proteins with unknown function from C. acetobutylicum and C. perfringens (34% identity with each of these proteins) (39, 48).
Figure 2: (A) Nucleotide sequences of promoter and translation initiation regions in the cir gene cluster. Putative –35, –10 and RBS sequences are underlined. Deduced amino acid sequences are indicated below the nucleotide sequences, gene names are given below the amino acid sequences. Putative start codons are indicated in boldface and are numbered when more than one possibility exists. Termination codons are underlined and in italic.

Circularin A production in a heterologous host.

The region encompassing cirABCDE was cloned in the broad host range vector pMG36c downstream of the constitutive lactococcal promoter P_{32}, creating pMGAE1. E. faecalis JH2-2 (pMGAE1) produced a low amount of bacteriocin and was resistant to circularin A (Fig. 5) but bacteriocin production was not stable. Three consecutive
Table 2: Characteristics of predicted proteins specified by the *C. beijerinckii* ATCC25752 cir gene clus

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene size (bp)*</th>
<th>kDa</th>
<th>pI</th>
<th>TM*</th>
<th>Homology</th>
<th>Putative fun</th>
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<tbody>
<tr>
<td>CfgR</td>
<td>774</td>
<td>30.4</td>
<td>6</td>
<td>0</td>
<td>response regulators</td>
<td>CirA precurs</td>
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<tr>
<td>CfgK</td>
<td>1275</td>
<td>49.8</td>
<td>6.8</td>
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<td>histidine kinases</td>
<td>secretion/im</td>
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<tr>
<td>Cfg01</td>
<td>597</td>
<td>23.2</td>
<td>9.8</td>
<td>5</td>
<td>AgrB regulatory protein</td>
<td></td>
</tr>
<tr>
<td>Cfg02</td>
<td>390</td>
<td>14.4</td>
<td>10.17</td>
<td>4</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>CirA</td>
<td>216</td>
<td>7.2</td>
<td>10.9</td>
<td>2</td>
<td>enterocin AS-48</td>
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</tr>
<tr>
<td>CirB</td>
<td>1743</td>
<td>68.8</td>
<td>9.4</td>
<td>11</td>
<td>AS-48B (19%)</td>
<td>secretion/im</td>
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<tr>
<td>CirC</td>
<td>555</td>
<td>20.9</td>
<td>10.1</td>
<td>4</td>
<td>AS-48C (21%)</td>
<td>maturation</td>
</tr>
<tr>
<td>CirD</td>
<td>663</td>
<td>25.7</td>
<td>6.4</td>
<td>0</td>
<td>ATP-binding proteins; AS-48D (31.6%)</td>
<td>secretion/im</td>
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<tr>
<td>CirE</td>
<td>147</td>
<td>5.7</td>
<td>10.6</td>
<td>2</td>
<td>AS-48DI (30%)</td>
<td>immunity</td>
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<td>CirG</td>
<td>1425</td>
<td>51.7</td>
<td>4.6</td>
<td>1</td>
<td>HlyD family of proteins/EmrA/BacG (17%)</td>
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<tr>
<td>CirH</td>
<td>744</td>
<td>27.6</td>
<td>6.1</td>
<td>0</td>
<td>ATP-binding proteins/BacH (40%)</td>
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<tr>
<td>CirI</td>
<td>1266</td>
<td>45.7</td>
<td>9.7</td>
<td>4</td>
<td>Duf214 domain/permease of ABC transporter/Bacl (32%)</td>
<td></td>
</tr>
</tbody>
</table>

*) When multiple putative starts were possible the longest product was used.

*) The pI of CirA is based on the linear unprocessed protein.

*) TM: Number of putative transmembrane sequences.

Homologues were identified using BLAST searches against the NCBI non-redundant protein database or by direct comparison to the proteins involved in enterocin AS-48 production. Within brackets: percentage of identity of the protein to its homologue involved in enterocin AS-48 production cycles of selection for bacteriocin production by overlay assay and subsequent clean-streaking of producing colonies yielded a strain that stably expressed a high level of circularin A (Fig. 5). The plasmid in this strain, designated pCir, was identical to pMGAE1 by restriction enzyme analysis. The high bacteriocin expression level was maintained upon plasmid isolation and re-introduction into *E. faecalis* JH2-2. The copy numbers of pCir and pMGAE1 were clearly reduced when compared to the copy number of the empty vector pMG36c (data not shown).

**Functional analysis of the circularin A gene cluster.**

In order to determine which genes are involved in the production of circularin A, pCir was used as a template to create in-frame single deletions of *cirA*, *cirB*, *cirC* or *cirD*. *E. faecalis* JH2-2 strains harboring the various plasmids had all lost the CirA+ phenotype, as revealed by colony overlay assays (Fig. 1). All strains remained resistant to circularin A as they grew on plates containing filter-sterilized culture supernatant (10% v/v) of *C. beijerinckii* ATCC 25752, while *E. faecalis* JH2-2 (pMG36c) did not (Fig. 1). These results indicate that each of the four gene products is required for the production of active circularin A. Removal of *cirE* from the *cirABCDE* cluster in pCir could not be achieved in several attempts using different cloning hosts (*E. faecalis* JH2-2; *E. faecalis* JH2-2 (pMGE)), while simultaneous deletion of *cirA* and *cirE* (pCirΔAE) was
Figure 3: Alignment of the putative ATP binding proteins involved in circularin A (CirD, CirH) and enterocin AS-48 (AS-48D, BacH) production with LolD, a protein involved in lipoprotein secretion. Identical residues are indicated by an asterisk. (:) and (.) indicate conserved and semiconserved substitutions of amino acids according to the CustalW grouping of amino acids, respectively. Dashes indicate gaps introduced in the sequence to maximize alignment. Walker A, Walker B and ABC transporter B motifs are indicated.

possible. This observation suggested that *cirE* is involved in bacteriocin immunity, an assumption that will be further discussed below. *E. faecalis* JH2-2 (pCirΔEAE) showed reduced sensitivity to circularin A present in filter-sterilized culture supernatant of *C. beijerinckii* ATCC 25752 but was clearly more resistant to circularin A than *E. faecalis* (pMG36c). To determine which gene(s) is involved in this partial resistance towards circularin A, single deletions of *cirB*, *cirC* or *cirD* were combined with a deletion in *cirE*. A mutation in either *cirB* or *cirD* in combination with *cirE* led to the loss of the CirR phenotype whereas cells carrying a pCir derivative with a deletion in *cirA* or *cirC* in combination with *cirE* remained partially resistant against circularin A (Fig. 1). These results indicate that both CirB and CirD are required for the partial resistance in the absence of CirE. To confirm this hypothesis three additional deletion constructs were made. *E. faecalis* JH2-2 (pCirΔACE), specifying only CirB and CirD, still showed partial
circular in A, single deletions of cirB, cirC or cirD were combined with a deletion in cirE.

Figure 4: Alignment of CirI with BacG (56), FtsX (6), LolC and LolE (37). The consensus motif G-X[9]-F-X[10]-G for ortholog group 3-1 type ABC transporters is indicated. Predicted transmembrane domains are indicated in bold. The region constituting the predicted DUF214 domain is indicated by a line above the sequences. Identical residues are indicated by an asterisk. (: ) and (. ) : conserved and semiconserved amino acid substitutions according to the CustalW grouping of amino acids, respectively. Gaps are introduced in the sequence to maximize alignment.

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Figure 4: Alignment of Cir with BacG (56), FtsX (6), LolC and LoE (37). The consensus motif G-X[9]-F-X[10]-G for ortholog group 3-1 type ABC transporters is indicated. Predicted transmembrane domains are indicated in bold. The region constituting the predicted DUF214 domain is indicated by a line above the sequences. Identical residues are indicated by an asterisk. (: ) and (. ) : conserved and semiconserved amino acid substitutions according to the CustalW grouping of amino acids, respectively. Gaps are introduced in the sequence to maximize alignment.
resistance to CirA. *E. faecalis* JH2-2 expressing only CirB (pCir\(\Delta\)ACDE) or CirD (pCir\(\Delta\)ABCE) was bacteriocin sensitive, confirming that both CirB and CirD are needed for the partial resistance phenotype in the absence of CirE.

Heterologous expression of circularin A in *L. lactis* NZ9000 was attempted but pCir or any other vector containing cirB did not give transformants while all control plasmids did. Apparently, an intact cirB gene is lethal to this host. Plasmids with a deletion in cirB could be stably maintained in *L. lactis*. *L. lactis* (pCir\(\Delta\)B) did not produce active CirA and was 2.5-fold more resistant to the bacteriocin than *L. lactis* (pNG8048e), a strain carrying an empty cloning vector.

**The cirE gene confers circularin A immunity.**

To prove that cirE can confer bacteriocin resistance independent of the combination cirB and cirD the gene was cloned downstream of the lactococcal promoter P\(\_32\) in pMG36e (pMG-E). Unlike *L. lactis* (pMG36e), *L. lactis* NZ9000 (pMG-E) was able to grow in a medium with culture supernatant (up to 50% v/v) of *C. beijerinckii* ATCC 25752 (CirA\(^+\)), as determined by serial dilution assay. *L. lactis* NZ9000 (pMG-E) also formed normal colonies on plates containing filter-sterilized *C. beijerinckii* ATCC 25752 culture supernatant (4% v/v), whereas the control strain did not grow at all. These results indicate that cirE alone gives rise to circularin A resistance. *E. faecalis* JH2-2 (pMG-E) was also immune to the bacteriocin present in *C. beijerinckii* ATCC 25752 supernatant, as determined in a plate assay. As mentioned above, initial attempts to remove cirE from cirABCDE, using either *E. faecalis* JH2-2 or *E. faecalis* JH2-2 (pMG-E) as cloning hosts, failed. This problem was circumvented by cloning cirE downstream of the P\(\_32\) promoter in pIL253. The resulting strain, *E. faecalis* JH2-2 (pIL-E), was immune to *C. beijerinckii* ATCC 25752 culture supernatant as determined in a plate assay and did not produce bacteriocin (Fig. 1) as determined by serial dilution and overlay assays. In *E. faecalis* JH2-2 (pIL-E) we were able to introduce the cirE deletion plasmid pCir\(\Delta\)E (CirA\(^+\)). *E. faecalis* JH2-2 (pIL-E, pCir\(\Delta\)E) was immune to *C. beijerinckii* ATCC 25752 culture supernatant and produced bacteriocin (Fig. 1). Attempts to introduce pCir\(\Delta\)E in *E. faecalis* JH2-2 alone were unsuccessful, indicating that cirE is required for proper bacteriocin immunity. The levels of resistance conferred by pMG-E or pIL-E in *E. faecalis* JH2-2, which are able to grow in medium containing filter-sterilized *C. beijerinckii* ATCC 25752 culture supernatant (at the most 3-6% v/v), are only 2- to 4-fold increased compared to the control strain carrying only pMG36e, able to grow in medium containing at the most 1.5% (v/v) of filter-sterilized *C. beijerinckii* ATCC 25752 culture supernatant. This level is lower than the resistance level of *E. faecalis* JH2-2 (pCir\(\Delta\)B) or *E. faecalis* JH2-2 (pCir\(\Delta\)D), both also only expressing cirE as a functional immunity system. Complementing pIL-E with pCir\(\Delta\)DE in *E. faecalis* JH2-2 did not restore the resistance level to that of *E. faecalis* JH2-2 (pCir\(\Delta\)D),
indicating that the lower resistance level is not due to the absence of auxiliary factors, not present on pMG-E/pIL-E. Taken together, these results confirm that CirE is the dedicated circularin A immunity protein and that expression of cirE alone is sufficient for immunity.

**DISCUSSION**

The region surrounding the structural gene cirA of the circular bacteriocin circularin A of *C. beijerinckii* ATCC 25752 encompasses eleven genes. Upstream of cirA there are four genes of which two could encode a two component regulatory system. Together with the presence of an AgrB homologue (Cfg01) this presents the possibility of regulation of bacteriocin expression. Two component systems are often involved in the regulation of bacteriocin expression and their genes are normally located near the bacteriocin operon (27). The homology to the Agr system of *Staphylococcus aureus*, which consists of a two component system, a processing protein (AgrB) and a signaling peptide (AgrD) involved in regulation of virulence factors (40, 61), suggests a similar regulatory mechanism although we could not identify an ArgD homologue in our sequence and have not further addressed the possible involvement of CfgR and CfgK in CirA expression.

We show here that the region cirABCDEGHI is involved in bacteriocin production and secretion. The genes cirABCDE represent the minimal region required for bacteriocin processing and secretion in the heterologous host *E. faecalis* JH2-2 as deletion of only a single gene from this cluster either causes loss of bacteriocin production or loss of cell viability. The genetic organization of the region cirABCDEGHI seems rather compressed as several genes overlap. This set up suggests that translational coupling, a gene regulatory mechanism often used in operons in which the stoichiometry of gene expression is important (36), may occur.
The minimal requirements for extracellular circularin A activity are production, processing, circularization and secretion of the bacteriocin, while the producer cell should be immune for the bacteriocin. All these features should in principle be encoded by cirABCDE. Here, we show that resistance against circularin A is acquired via at least two independent systems. First, expression of cirE confers a certain level of immunity to the expressing strain, which is essential for the bacteria to be able to produce and withstand CirA. CirE has a very high pI and contains two possible transmembrane helices, which make a membrane localization of the protein very likely. Its small size, high pI and two predicted transmembrane helices are characteristics that CirE has in common with AS-48D1, the immunity protein of the circular bacteriocin enterocin AS-48, and with the proteins PepI, Ecil, LasJ and DviA, that have all been shown or postulated to be involved in immunity to the unrelated bacteriocins Pep5, epicidin 280, lactocin S and divergicin A, respectively (18, 41, 44, 50, 59). The immunity mechanism of these proteins is unknown but PepI has been suggested to inhibit pore formation by Pep5 (46). The second system conferring reduced sensitivity to CirA depends on the combined activity of CirB and CirD. Together these proteins form a putative ABC transporter in which CirB is the transporter and CirD provides the nucleotide-binding domain. The putative transporter CirBD confers a basal level of CirA resistance, which is, however, insufficient to support bacteriocin production by the heterologous host we used: a viable clone of E. faecalis JH2-2 (pCirE) could not be obtained. CirBD (most likely) also function in CirA secretion, as ABC transporters are often implicated in bacteriocin secretion (10). The fact that proteins required for secretion of a bacteriocin can be involved in resistance has been shown for McbE and McbF, which are involved in microcin B17 production (13). The secretion proteins of the lantibiotic nisin were suggested to fulfill a similar role but involvement of NisI and/or NisFEG, via a regulatory loop inducing expression of the respective genes, can not be excluded (29, 43). Resistance is most probably obtained by the pumping out of the bacteriocin (42, 43, 46). In conclusion, CirBD confer low level resistance by virtue of their ability to secrete CirA while CirE shows structural homology to other bacteriocin immunity proteins, which identifies it as the dedicated CirA immunity protein.

Based on homology studies, CirGHI could constitute another transporter. CirG probably has an auxiliary function as it is homologous to the HlyD family of proteins, many of which are accessory proteins in the export of drugs or toxic proteins such as hemolysin (52), lactococcin A (53) and colicin V (15). CirH and CirI probably form an ABC-transporter of the LolCDE type (60): CirH is homologous to LolD while CirI is homologous to both LolC and LolE. The CirGHI homologues in the enterocin AS-48 system (BacGHI/AS-48FGH) enhance the expression of enterocin AS-48 (56) and the resistance towards exogenous enterocin AS-48 (8), roles we have not investigated for CirGHI yet. The homology to the LolCDE system, furthermore, suggests that active
transport, perhaps from the outer leaflet of the membrane as shown for LolCDE (60), is involved in this enhancing effect by making more bacteriocin available. The NisFEG system fulfills such a function in enhancement of nisin secretion (43) but this system is not very homologous to CirGHI/LolCDE.

No experimental evidence has been obtained to identify the protein(s) involved in the processing and/or circularization of the CirA prepeptide. As CirBD together form a putative ABC-transporter and cirE confers bacteriocin immunity, the essential protein CirC is a likely candidate to perform this function(s), either alone or together with CirB and/or CirD. This notion seems to be supported by the fact that the only other CirC homologue is AS-48C, encoded by the enterocin AS-48 gene cluster of *E. faecalis*. In conclusion, we have identified five essential genes for circularin A production and have shown that three of these genes (*cirBDE*) are involved in bacteriocin resistance. Future studies will be performed to determine the mechanism of circularization and the possible role of CirC therein.

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