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Functional Analysis of the Gene Cluster Involved in Production of the Bacteriocin Circularin A by *Clostridium beijerinckii* ATCC 25752

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A region of 12 kb flanking the structural gene of the cyclic antibacterial 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 organized in overlapping open reading frames. Heterologous expression of circularin A in *Enterococcus faecalis* was achieved, and five genes were identified as 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.

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 a few antimicrobial peptides of 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*, *Clostridium botulinum*, and *Clostridium 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, nonmodified, head-to-tail-ligated cyclic antibacterial 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 bacteriocin action on cells 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 among bacteriocin immunity proteins, even those that are involved in immunity against bacteriocins of the same class.

In this study we identified the genes required for functional heterologous expression of circularin A and showed 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, this study will further the field of clostridial bacteriocins and that of class V (circular) bacteriocins in particular.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source[b]
C. beijerinckii ATCC 25752	Circularin A producer	NIZO
E. faecalis JH2-2	Plasmid-free derivative of *E. faecalis* JH-2	20
L. lactis NZ9000	Plasmid-free derivative of *L. lactis* MG1363, *pepN*::*nisRK*	30
Lactobacillus sake ATCC 15521	Bacteriocin indicator	lab collection
E. coli Top10	*F′ mcrA* (*mrr-hsdRMS-mcrBC*) ϕ80*lacZM15 lacX74 recA1 deoR araD139* (*ara-leu*)*7697 galU galK rpsL* (*Str*^{r}) *endA1 nupG*	Invitrogen
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	17
Plasmids		
pMG36c	Cm^r, pWV01-based cloning vector carrying the strong lactococcal promoter P_{32}	Laboratory collection
pMG36e	Em^r, gene expression vector carrying P_{32}	57
pCR21	Amp^r Km^r	Invitrogen
pCRAE	Amp^r Km^r	This study
pIL253	Em^r, theta replicating cloning vector	49
pIL-E	Em^r, pIL253 derivative with *cirE* under control of P_{32}	This study
pIL-P32	Em^r, pIL253 derivative with P_{32} promoter	This study
pMGAE1	Cm^r, pMG36c derivative with *cirABCDE* under control of P_{32}	This study
pCir	Cm^r, derivative of pMGAE1 overexpressing circularin A	This study
pCirΔA	Cm^r, pCir Δ*cirA*	This study
pCirΔB	Cm^r, pCir Δ*cirB*	This study
pCirΔC	Cm^r, pCir Δ*cirC*	This study
pCirΔD	Cm^r, pCir Δ*cirD*	This study
pCirΔE	Cm^r, pCir Δ*cirE*	This study
pCirΔAE	Cm^r, pCir Δ*cirA* Δ*cirE*	This study
pCirΔBE	Cm^r, pCir Δ*cirB* Δ*cirE*	This study
pCirΔCE	Cm^r, pCir Δ*cirC* Δ*cirE*	This study
pCirΔDE	Cm^r, pCir Δ*cirD* Δ*cirE*	This study
pCirΔACE	Cm^r, pCir Δ*cirA* Δ*cirC* Δ*cirE*	This study
pCirΔABCE	Cm^r, pCir Δ*cirA* Δ*cirB* Δ*cirC* Δ*cirE*	This study
pCirΔACDE	Cm^r, pCir Δ*cirA* Δ*cirC* Δ*cirD* Δ*cirE*	This study
pMG-E	Em^r, pMG36e derivative with *cirE* under control of P_{32}	This study

[a] Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Km^r, kanamycin resistance.
[b] NIZO, NIZO Food Research Ede, The Netherlands; Invitrogen, Breda, The Netherlands.

MATERIALS 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, Mich.). 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. Twofold-diluted M17 broth (Difco) with a final concentration of 1.9% β-glycerophosphate (Merck) and 0.5% glucose (G[1/2]M17) 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 h in tryptone-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 (Sigma) were used at 100 and 10 μg/ml, respectively, for *E. coli*. Chloramphenicol and erythromycin (Sigma) were used at 5 μg/ml each for *L. lactis* NZ9000 and at 20 and 2 μg/ml, respectively, for *E. faecalis* JH2-2. When used together, chloramphenicol and erythromycin were employed at 2.5 μg/ml each for *Lactobacillus saké* ATCC 15521 or at 10 μg/ml (chloramphenicol) and 2 μg/ml (erythromycin) for *E. faecalis* JH2-2.

Nucleotide sequencing. Inverse PCR techniques with 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 with the High Pure PCR product purification kit of Roche (Roche Diagnostics GmbH, Mannheim, Germany). Sequencing was performed with indodicarbocyanine-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 and 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 microtiter plates. To 50 μl of serially diluted, bacteriocin-containing samples, 150 μl of medium containing the indicator strain *Lactobacillus saké* ATCC 15521(pMG36e; pMG36c), diluted 100-fold from a stationary-phase overnight culture, was added unless mentioned otherwise. Resistance to bacteriocin was determined by plating strains on plates containing 4 or 10% (vol/vol) filter-sterilized *C. beijerinckii* ATCC 25752 supernatant containing circularin A. Alternatively, a critical-dilution assay was performed with the strain of interest as an indicator strain. In these critical-dilution assays, 100 μl of bacteriocin-containing sample was mixed with 100 μl of freshly diluted (1,000-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) with 1% glycine (Merck). *E. faecalis* JH2-2 was transformed as described previously (47; http://w3.ouhsc.edu/enterococcus) with 8% glycine. After transformation, both strains were plated on G[1/2]M17 medium containing 0.5 M sucrose and the appropriate antibiotics. Plasmids from *L. lactis* NZ9000, *Escherichia coli* DH5α, and *E. faecalis* JH2-2 were isolated according to Birnboim (3), with the following modifications for *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 RNase (0.5 mg/ml; Sigma) treatment, further purified with the High Pure PCR product purification kit (Roche Diagnostics GmbH).

Cloning of the circularin A determinant. The region encompassing *cirA* to *cirE* was amplified with primers located just upstream of *cirA* (B51426, 5′-ACGCG TCGACTCATGAGTTTTTCAAAAGGAGGTGATTAATT ATGTTTTTATT GCAGG-3′) and downstream of *cirE* (B51427, 5′-CGCGGATCCGTCGACCT CTCCCACTTTAACATTAGTTATTGCTC-3′). *Sal*I-*Rca*I and *Bam*HI-*Sal*I sites, respectively, in the two primers are underlined. All enzymes (Roche) were

used according to the manufacturer's instruction. The PCR product was cloned with the Zero-Blunt Topo PCR cloning kit (Invitrogen, Breda, The Netherlands), creating pCRAE. The plasmid pCRAE was digested with *Spe*I and *Xho*I. The fragment carrying *cirA* was ligated into pMG36c digested with *Xba*I and *Sal*I, 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 with 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′-AGTATGG CAAGAGCTATAGC-3′ and 5′-CACGCCTAGTGCTCCTGC-3′ for Δ*cirA*, 5′-TAATTATGCCTGTATCATACC-3′ and 5′-CCAAGAGTTATAGTTTGAGT CG-3′ for Δ*cirB*, 5′-GTGCACATAGGTAGGATTTTAAG-3′ and 5′-GAAAC ATTCC AACAATAATACC-3′ for Δ*cirC*, and 5′-GAACTTAATCTAGTTAA CGGAAG-3′ and 5′-AGTTATCTCTAGCATAGGCTTC-3′ for Δ*cirD*.

Each PCR product was kinase treated with T4 polynucleotide kinase (Amersham Pharmacia Biotech) in T4 ligase buffer (Roche Diagnostics GmbH) and subsequently self-ligated with T4 ligase, creating the plasmids pCirΔA, pCirΔB, pCirΔC, and pCirΔD. Derivatives of pCir with a deletion of *cirE* and a deletion of one of the other genes *cirABCD*, pCirΔAE, pCirΔBE, pCirΔCE, or pCirΔDE, were made by the same method with the primers 5′-CATATATTCTACTACC TTTC-3′ and 5′-GTAATTAAAGGCTCTAATAAG-3′ for Δ*cirE* and the plasmids carrying the respective single deletions as templates in the PCR. A plasmid with a triple deletion, pCirΔACE, was constructed likewise by deleting *cirC* with pCirΔAE as a template and the primers used for the single deletion of *cirC*. Based on pCirΔACE, pCirΔABCE and pCirΔACDE were made with the primers employed for the single knockouts of *cirB* and *cirD*, respectively. All plasmids were isolated with *E. faecalis* JH2-2 as the cloning host.

The *cirE* gene was cloned behind the lactococcal chromosomal P_{32} promoter by digesting the PCR product obtained with primers B51426 and B51427 and *Hpa*I and *Sal*I and ligating the *cirE*-carrying fragment into *Sma*I- and *Sal*I-digested pMG36e, leading to pMG-E. Colonies obtained after transformation of *L. lactis* NZ9000 were replica streaked onto G[1/2]M17 plates with 4% (vol/vol) *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 pCirΔE, a fragment with the immunity gene *cirE* behind the P_{32} promoter was first cloned in pIL253 to avoid possible lethal effects of bacteriocin expression without immunity. This was done by digesting pMG-E with *Eco*RI and *Sal*I and ligating the *cirE*-carrying fragment into pIL253 digested with the same enzymes. The resulting plasmid (pIL-E) was introduced into *E. faecalis* JH2-2. pCirΔE was subsequently made with pCir as a template and the appropriate primers. *E. faecalis* JH2-2(pIL-E) was used as the host for construction of pCirΔE.

Computational analyses. Open reading frames were identified with the Glimmer 2.0 program (6). Predictions by the Glimmer 2.0 program were checked manually for validity. Homology comparisons were performed with the basic logical alignment tool (Blast) as described by Altschul et al. (1). Blast searches were performed against the NCBI nonredundant protein database and the NCBI microbial genomes database (http://www.ncbi.nlm.nih.gov/BLAST/). Homologies with conserved domains from the Pfam database (http://www.sanger.ac.uk /Software/Pfam/) (2) were also identified with Blast searches. Putative signal peptides were identified with signalP (http://www.cbs.dtu.dk/services/SignalP/) (38). Putative transmembrane helices were identified with the TMHMM2.0 program (http://www.cbs.dtu.dk/services/TMHMM-2.0/) (28). Dyad symmetries, isoelectric points, and molecular weights were determined with the program Clonemanager 4 (SEcentral; Scientific & Educational Software). Sequence alignments were performed with the ClustalW program available at http://www2.ebi.ac.uk /clustalw/ (54).

Nucleotide sequence accession number. The *cirA* sequence is available under GenBank accession number AJ566621.

RESULTS

Sequence analysis of the region encompassing the structural gene of circularin A. 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 12 open reading frames (ORFs), including *cirA* (Fig. 1 and 2). 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 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, both 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 [7] and LolC and LolE [37]), CirI contains four putative transmembrane domains, typical of 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 response regulators and histidine kinases, respectively, 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), 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).

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 small amount of bacteriocin and was resistant to circularin A (Fig. 5), but bacteriocin production was not stable. Three consecutive 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 reintroduction into *E. faecalis* JH2-2. The copy numbers of pCir and pMGAE1 were clearly

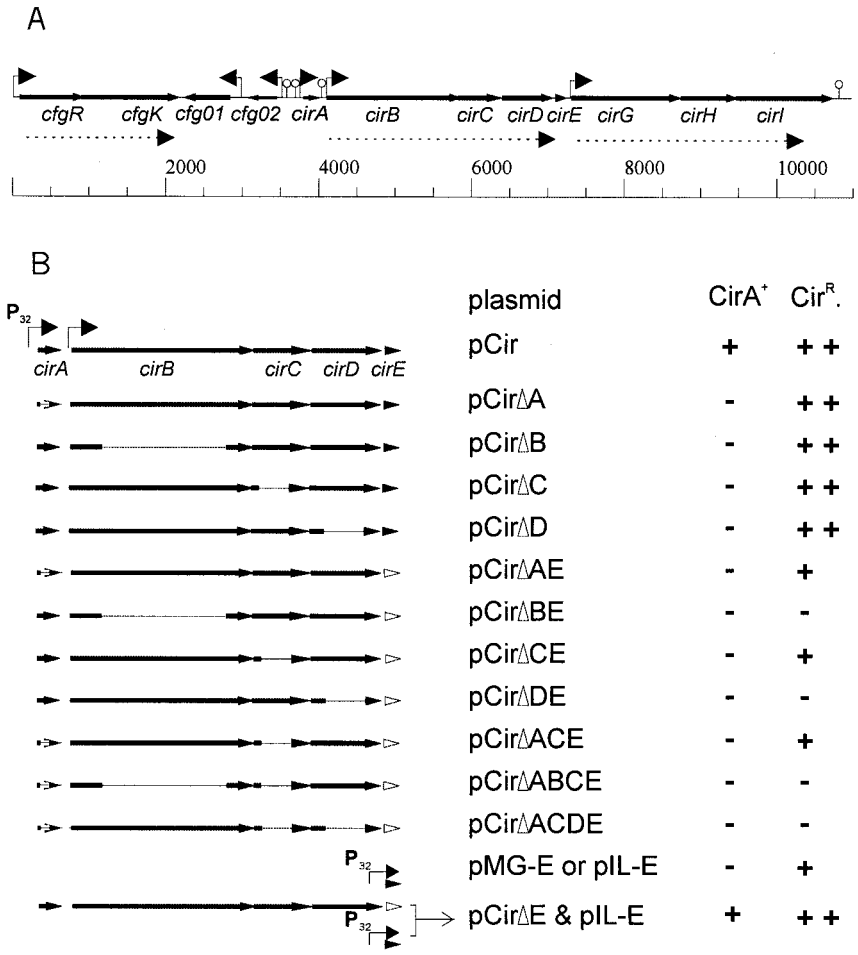

FIG. 1. (A) Physical map of the region surrounding the circularin A structural gene *cirA* of *C. beijerinckii* ATCC 25752. Solid arrows indicate genes; bent arrows show putative promoters; lollipops represent predicted regions of dyad symmetry ($\Delta G^0 < -10$ kcal/mol); dotted arrows show possible polycistronic messengers. Map units are base pairs. (B) Schematic representation of the *cir* DNA fragments in the indicated plasmids and locations 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; Cir^R, circularin A resistance, denoted as ++, full protection against CirA [>24-fold increase relative to *E. faecalis* JH2-2(pMG36c)]; +, partial protection [2- to 16-fold increase relative to *E. faecalis* JH2-2(pMG36c)]; −, sensitive.

reduced 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 all lost the $CirA^+$ phenotype, as revealed by colony overlayer assays (Fig. 1). All strains remained resistant to circularin A, as they grew on plates containing filter-sterilized culture supernatant (10%, vol/vol) 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 with different cloning hosts [*E. faecalis* JH2-2 and *E. faecalis* JH2-2(pMGE)], while simultaneous deletion of *cirA* and *cirE* (pCirΔAE) was possible. This observation suggested that *cirE* is involved in bacteriocin immunity, an assumption that will be discussed further below. *E. faecalis* JH2-2(pCirΔAE) 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*, and *cirD* were combined with a deletion in *cirE*. A mutation in either *cirB* or *cirD* in combination with deletion of *cirE* led to the loss of the circularin A-resistant phenotype, whereas cells carrying a pCir derivative with a deletion in *cirA* or *cirC* in combination with *cirE* remained partially resistant to circularin A (Fig. 1). These results indicate that both CirB and CirD are required for 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 resistance to CirA. *E. faecalis* JH2-2 expressing only CirB (pCirΔACDE) or CirD (pCirΔABCE) was bacteriocin sensitive, confirming that both CirB and CirD

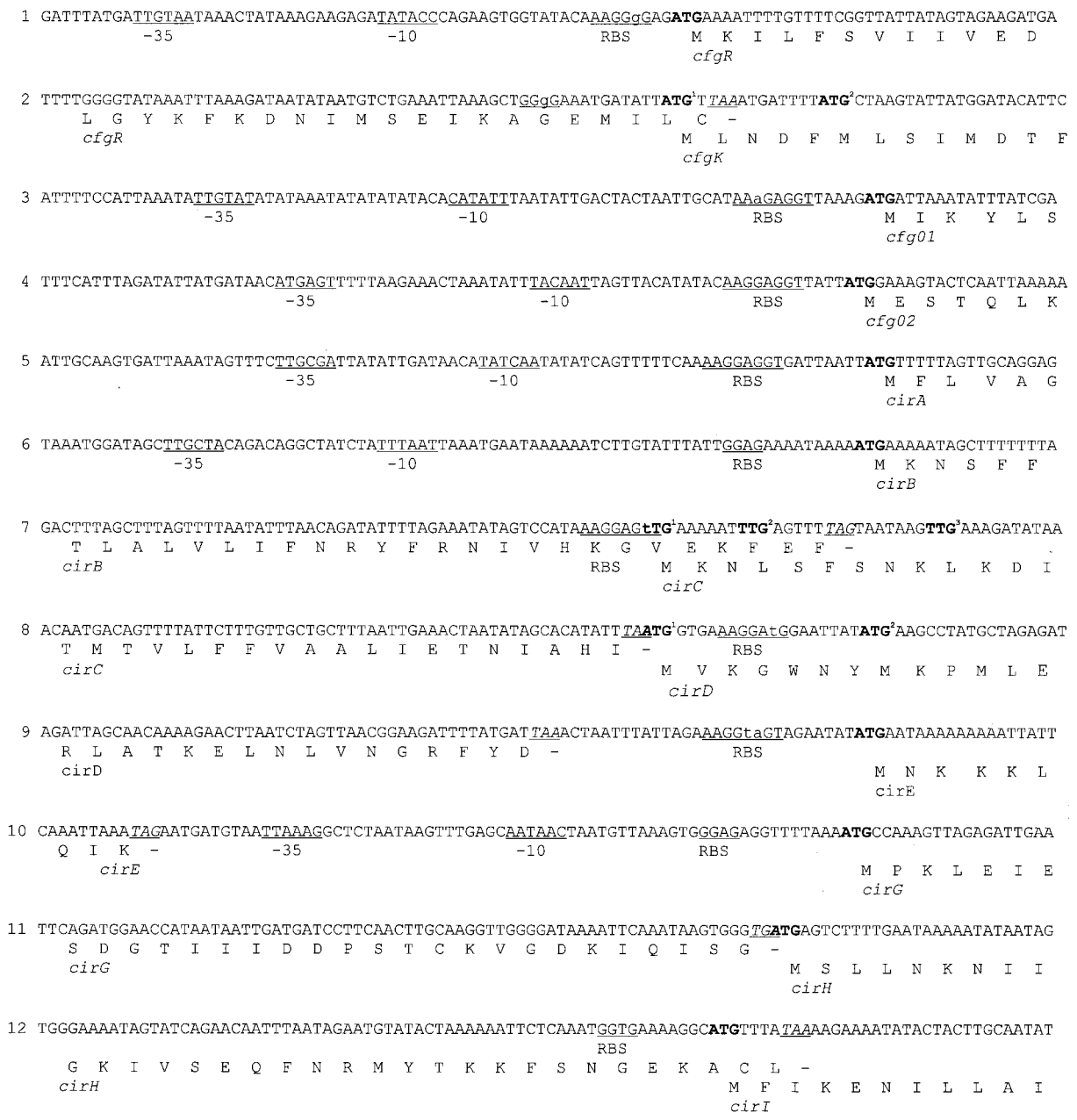

FIG. 2. (A) Nucleotide sequences of promoter and translation initiation regions in the *cir* gene cluster. Putative −35, −10, and ribosome-binding site (RBS) sequences are underlined. Deduced amino acid sequences are indicated below the nucleotide sequences, and gene names are given below the amino acid sequences. Putative start codons are indicated in boldface and are numbered when more than one possibility exists. Termination codons are underlined and in italic.

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 and 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*(pCirΔB) 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.

***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%, vol/vol) 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%, vol/vol), whereas the

TABLE 2. Characteristics of predicted proteins specified by the *C. beijerinckii* ATCC 25752 *cir* gene cluster

Protein	Gene size[a] (bp)	Protein				
		Size (kDa)	pI	TM[b]	Homology[c]	Putative function
CfgR	774	30.4	6	0	Response regulators	
CfgK	1,275	49.8	6.8	7	Histidine kinases	
Cfg01	597	23.2	9.8	5	AgrB regulatory protein	
Cfg02	390	14.4	10.17	4	Unknown	
CirA	216	7.2	10.9[d]	2	Enterocin AS-48	CirA precursor
CirB	1,743	68.8	9.4	11	AS-48B (19%)	Secretion/immunity
CirC	555	20.9	10.1	4	AS-48C (21%)	Maturation
CirD	663	25.7	6.4	0	ATP-binding proteins; AS-48D (31.6%)	Secretion/immunity
CirE	147	5.7	10.6	2	AS-48D1 (30%)	Immunity
CirG	1,425	51.7	4.6	1	HlyD family of proteins/EmrA/BacG (17%)	
CirH	744	27.6	6.1	0	ATP-binding proteins/BacH (40%)	
CirI	1,266	45.7	9.7	4	Duf214 domain/permease of ABC transporter/BacI (32%)	

[a] When multiple putative starts were possible, the longest product was used.
[b] TM, number of putative transmembrane sequences.
[c] Homologues were identified by using Blast searches against the NCBI nonredundant protein database or by direct comparison to the proteins involved in enterocin AS-48 production. The identity of the protein to its homologue involved in enterocin AS-48 production is shown in parentheses.
[d] The pI of CirA is based on the linear unprocessed protein.

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* with either *E. faecalis* JH2-2 or *E. faecalis* JH2-2(pMG-E) as the cloning host 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

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                                                                  Walker A
CirH            ---MSLLNKNIIEMKNIVKSFYIGTPNQLDILKNIDITIKEGEFISIVGASGSGKSTLMN 57
BacH            ----------MIELNKINK-YYANEEESLHVLKDIHLSIKKGEMIAIMGPSGSGKSTLIN 49
LolD            ------MNKILLQCDNLCKRYQEGS-VQTDVLHNVSFSVGEGEMMAIVGSSGSGKSTLLH 53
CirD            MVKGWNYMKPMLEITNLSFSYSEET----EVLKNLDFTLNEKEILCIKGPNGAGKTTFLK 56
AS-48D          --------MKKITINNLSFYYESKD---TMVFDRLSLEFSSEKIYALVGSNGVGKTTLLN 49
                           :   ::   :         ::. : : . . :: .: *..* **:*:::

CirH            IIGALDRQTSGNYILDNFNMNEVSDDGLSQIRNKKIGFVFQTFNLIPRSTALKNVELPML 117
BacH            LLGFIDRKFEGEYLFEGRSLVNTADDILSKIRNQTVGFIFQNFSLIESNTVYENVELPLL 109
LolD            LLGGLDTPTSGDVIFNGQPMSKLSSAAKAELRNQKLGFIYQFHHLLPDFTALENVAMPLL 113
CirD            ILCGLIRINSCNLKYMGQ------KNKLRNIKDD-IAYVPSDPYLYTKLTGMENLELICD 109
AS-48D          ILSGIYQPTGGTIEYDGT-------LYTEKVTKEKVAFIPYKTKLYPYLDVFDHIKLIAE 102
                ::  :           .            :: .. :.::     *       .:: :

                                                  ABC-trans.
CirH            YA--GVDRKERLERAESLLNLVGMEDRITHVPNELSGGQKQRVAIARALANDPSIILADE 175
BacH            YN--GLSPFKTKEKVFSVLDRVGLKGKEYKYPKQLSGGQQQRVAIARALINHPKFIIADE 167
LolD            IG--KKKPAEINSRALEMLKAVGLDHRANHRPSELSGGERQRVAIARALVNNPRLVLADE 171
CirD            IW--KEDKKKFMLTSMELANFFNLEEDLNTYVEDYSLGMKHKLYLIGMLSRNTKIIIMDE 167
AS-48D          LWGIKTDYLEYKRKVLEYCNRLNLD-YYNKRVESYSTGMEYKLYISLMLARDVSLVLLDE 161
                      .  :      .  . ..:.       .. * * . :: :   * ..  ::: **

                Walker B
CirH            PTGALDSSTGRVVMDLFHKVHELEGKTIILITHNQELAEETERIITMKDGKIVSEQFNRM 235
BacH            PTGALDTHTSEEIMKLFTTLNKEDDVTLIMVTHNPEVVPYCHRLITIRDGAIIED----- 222
LolD            PTGNLDARNADSIFQLLGELNRLQGTAFLVVTHDLQLAKRMSRQLEMRDGRLTAE----- 226
CirD            PLTALDIQSQNIAIKMFHEYVQLNKSIIFVSHINDLITRLATKELNLVNGRFYD------ 221
AS-48D          PFTMLDKKSRYLAMDLIKEK---KIITIFSSHQKDIVEYLSNDIINLDKLKGVNL----- 213
                *   **  .    :.::      .   ::    .  :       : : .

CirH            YTKKFSNGEKACL 248
BacH            --KELVQ------ 227
LolD            --LSLMGAE---- 233
CirD            ------------- 
AS-48D          --KNYEKN----- 219
```

FIG. 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. Colons and periods indicate conserved and semiconserved amino acid substitutions, respectively, according to the ClustalW grouping of amino acids. Dashes indicate gaps introduced in the sequence to maximize alignment. Walker A, Walker, and ABC transporter B motifs are indicated.

FIG. 4. Alignment of CirI with BacG (56), FtsX (6), and 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, whereas colons and periods indicate conserved and semiconserved amino acid substitutions, respectively, according to the ClustalW grouping of amino acids. Gaps were introduced in the sequence to maximize alignment.

(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 pCirΔE ($CirA^+$). *E. faecalis* JH2-2(pIL-E, pCirΔE) was immune to *C. beijerinckii* ATCC 25752 culture supernatant and produced bacteriocin (Fig. 1). Attempts to introduce pCirΔE 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 and pIL-E in *E. faecalis* JH2-2, which is able to grow in medium containing filter-sterilized *C. beijerinckii* ATCC 25752 culture supernatant (at the most 3 to 6%, vol/vol), were only two- to fourfold increased compared to the control strain carrying only pMG36e, able to grow in medium containing at the most 1.5% (vol/vol) filter-sterilized *C. beijerinckii* ATCC 25752 culture supernatant. This level is lower than the resistance level of *E. faecalis* JH2-2(pCirΔB) or *E. faecalis* JH2-2(pCirΔD), both also expressing only *cirE* as a functional immunity system. Complementing pIL-E with pCirΔDE in *E. faecalis* JH2-2 did not restore the resistance level to that of *E. faecalis* JH2-2(pCirΔD), indicating that the lower resistance level is not due to the absence of auxiliary factors not present on pMG-E or

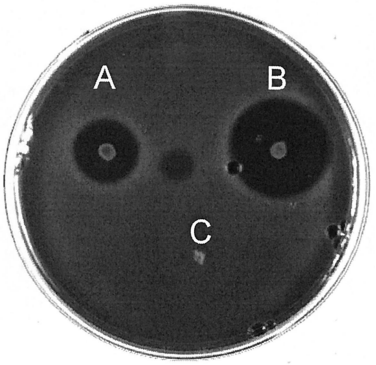

FIG. 5. Heterologous production of circularin A by *E. faecalis* JH2-2, as visualized in a colony overlayer assay with *Lactobacillus saké* ATCC 15521 as the indicator strain. (A) *E. faecalis* JH2-2(pMGAE1); (B) *E. faecalis* JH2-2(pCir); (C) *E. faecalis* JH2-2(pMG36c).

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 11 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 causes loss of either bacteriocin production or 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 to the bacteriocin. All these features should in principle be encoded by *cirABCDE*. Here, we show that resistance to 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 and contains two possible transmembrane helices, which make membrane localization of the protein very likely. Its small size, high isoelectric point, 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, EciI, LasJ, and DviA, which 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 that we used; a viable clone of *E. faecalis* JH2-2(pCirΔE) 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, cannot 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 to 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 or 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 genes essential 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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