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Amylocyclicin, a Novel Circular Bacteriocin Produced by Bacillus amyloliquefaciens FZB42

Romy Scholz, Joachim Vater, Anto Budiharjo, Zhiyuan Wang, Yueqi He, Kristin Dietel, Torsten Schwecke, Stefanie Herfort, Peter Lasch, Rainer Borriss

Institut für Biologie/Bakteriengenetik, Humboldt Universität Berlin, Berlin, Germany; Institut für Chemie, Technische Universität Berlin, Berlin, Germany; Key Laboratory of Plant Pathology of the Ministry of Education, Yunnan Agricultural University, Kunming, China; ABI-TEP GmbH Berlin, Berlin, Germany; Robert-Koch-Institut Berlin, Berlin, Germany

Bacillus amyloliquefaciens FZB42 is a Gram-positive plant growth-promoting bacterium with a highly impressive capacity to synthesize ribosomally produced antimicrobial metabolites (1). The compound displays high antibacterial activity against closely related Gram-positive bacteria. The genomic sequence of Bacillus subtilis showed that the FZB42 genome contains a cluster of six genes covering 4,490 bp that is responsible for the production of the antibacterial compound, here designated amylocyclicin, with a molecular mass of 6,381 Da. Peptide sequencing of the fragments obtained after tryptic digestion of the purified peptide revealed posttranslational cleavage of an N-terminal extension and head-to-tail circularization of the novel bacteriocin. Homology to other putative circular bacteriocins in related bacteria indicates that this type of peptide is widespread among the Bacillus/Paenibacillus taxon.

**Materials and Methods**

Cultivation of FZB42 and mutant strains for molecular biological studies. The B. amylo liquefaciens strains and plasmids used in this study are listed in Table 1. Bacillus and indicator strains were cultivated routinely on lysogeny broth (LB) medium solidified with 1.5% agar. For production of amylocyclicin, a medium containing 40 g of soy peptone, 40 g of dextrin, 1.8 g of KH₂PO₄, 4.5 g of K₂HPO₄, 0.3 g of MgSO₄ · 7H₂O, and 0.2 ml KellyT trace metal solution per liter was used. KellyT trace metal solution consists of 25 g of K₂H₂PO₄, 4.5 g of K₂HPO₄, 0.3 g of MgSO₄ · 7H₂O, and 0.2 ml KellyT trace metal solution per liter was used. KellyT trace metal solution consists of 25 mg of EDTA, 0.5 mg of ZnSO₄ · 7H₂O, 3.67 mg of CaCl₂ · 2H₂O, 1.25 g of MnCl₂ · 4H₂O, 0.25 g of CoCl₂ · 6H₂O, 0.25 g of ammonium molybdate, 2.5 g of FeSO₄ · 7H₂O, and 0.1 g of CuSO₄ · 5H₂O in 500 ml of H₂O adjusted to pH 6 with NaOH.

Liquid production medium (50 ml) in a 500-ml glass was inoculated with 500 μl of a LB overnight culture and shaken for 6.5 h until the optical density at 600 nm (OD₆₀₀) reached 8.5 (end of log phase), and the cells were pelleted by centrifugation at 14,000 rpm for 5 min.

Cultivation of FZB42 and mutant strains for the preparation of surface extracts. For the preparation of surface extracts, the wild-type strain B. amylo liquefaciens FZB42 and mutant strain R56 were grown in half-

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TABLE 1 Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td></td>
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</tr>
<tr>
<td>CU1065</td>
<td>168 trpC2 attSPB</td>
<td>2</td>
</tr>
<tr>
<td>HB0042</td>
<td>168 trpC2 attSPB sigW:kan</td>
<td>2</td>
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<tr>
<td>HB10102</td>
<td>168 sigW:mls</td>
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<tr>
<td>HB00608</td>
<td>CU1065 fosB:Cm</td>
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<tr>
<td>HB101031</td>
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</tr>
<tr>
<td>HB101013</td>
<td>CU1065 ydiST::kan</td>
<td>2</td>
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<td><strong>Bacillus megaterium 7A/1</strong></td>
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<tr>
<td>FZB42</td>
<td>Type strain B. amylovorans subsp. plantarum</td>
<td>DSMZ (DSM23117), BGSC (10A6)</td>
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<td>CH5</td>
<td>FZB42 sfp:ermAM yceE:Cm</td>
<td>6</td>
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<tr>
<td>RSpMarA2</td>
<td>Insertion of pMarA in CH5 degU::kan</td>
<td>This work</td>
</tr>
<tr>
<td>RS6</td>
<td>sfp:ermAM lac::Cm, deficient in lipopeptides, polypeptides, and bacilysin</td>
<td>6</td>
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<td>RS16</td>
<td>RS6 ΔacnE::spc</td>
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<td>RS17</td>
<td>RS6 ΔacnA::spc</td>
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</tr>
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<td>RS6 ΔacnB::spc</td>
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<td>RS6 ΔacnC::spc</td>
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<td>CH5 ΔacnBACDEF</td>
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<td>pMarA</td>
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<tr>
<td>pC333</td>
<td>Plasmid with spectrinocin resistance cassette</td>
<td>T. Msadek, Institute Pasteur, Paris, France</td>
</tr>
<tr>
<td>pGEM-T_km</td>
<td>pGEM-T containing kanamycin resistance cassette</td>
<td>This work</td>
</tr>
<tr>
<td>pGEM-T_kmR</td>
<td>pGEM-T_km with right flanking site of acn cluster</td>
<td>This work</td>
</tr>
<tr>
<td>pGEM-T_kmRL</td>
<td>pGEM-T_kmR with right and left flanking sites of acn cluster</td>
<td>This work</td>
</tr>
</tbody>
</table>

Concentrated potato dextrose bouillon (PDB) medium (Carl Roth GmbH) for 24 h. For preculture, 10 ml LB was inoculated with bacteria from a freshly grown agar plate, and the plate was shaken overnight at 200 rpm and 30°C. The main culture of 120 ml half-concentrated PDB medium was inoculated with bacteria from the preculture to an end concentration of 1.0 × 10^8 CFU/ml and shaken at 120 rpm and 30°C. The culture was harvested after 24 h and centrifuged at 4,000 rpm and 3°C. The pellet was stored at −80°C. For preparation of surface extracts, cells were extracted twice with 50% aqueous acetonitrile–0.1% trifluoroacetic acid. The main culture of 120 ml half-concentrated PDB medium (Carl Roth GmbH) for 24 h. For preparation of surface extracts, cells were extracted twice with 50% aqueous acetonitrile–0.1% trifluoroacetic acid. Surface extracts were stored at −20°C prior to use.

**Strain construction.** The media and buffers used for DNA transformation of Bacillus cells were prepared as described by Kunst and Rapoport (16). Competent cells were prepared as previously described (17). Mutants were generated by transformation of the FZB42 derivatives with linearized, integrative plasmids or splicing by overhang extension (SOE) PCR fusion products containing resistance cassettes flanked by DNA regions homologous to the FZB42 chromosome. The oligonucleotides used for strain construction are listed in Table S1 in the supplemental material. Spectominycin (90 µg/ml) was used for the selection of transforrants.

Deletion of the whole acn gene cluster, yielding mutant WT06, was obtained after transformation of the FZB42-derived strain CH5 (Δsfp ΔyczE) with a linearized, integrative plasmid containing a neomycin resistance cassette flanked by DNA regions homologous to the FZB42 chromosome. A fragment containing the neomycin resistance cassette was amplified with the primers kmpamarA-1 and kmpamarA-2 (see Table S1 in the supplemental material) using plasmid pMarA as the template. This fragment was inserted into pGEM-T Easy (pGEM-T) to create pGEM-T_km. A fragment spanning the right side of the acn gene cluster was amplified using the primers yjcK-1-sacI and yjcK-2 and ligated into the SacI and SapI restriction sites of pGEM-T_km, yielding pGEM-T_kmR. A fragment spanning the left side of the acn gene cluster was amplified with the primers Guac-N-1-sphI and Guac-N-2-sphI and inserted into the transformants Kanr and Erms at the restrictive temperature for plasmid replication (30°C) and Kanr and Erms at the restrictive temperature for plasmid replication (48°C). To verify that these transformants contained the original intact plasmid, the plasmid was extracted and used to transform Escherichia coli DH5α. Plasmid DNA was extracted from E. coli DH5α and subjected to restriction endonuclease analysis with EcoRI. The restriction was then analyzed by agarose gel electrophoresis. For inducing transposition, isolated clones were grown overnight in liquid LB medium at 37°C, and then portions of each culture were plated on either LB, LB and kanamycin (5 µg/ml), or LB and erythromycin-lincomycin (1 µg/ml/25 µg/ml) and incubated at the nonpermissive temperature for plasmid replication (48°C) to select for transposants.
Selection for the loss of antibacterial activity using a bioassay (see below) with *Bacillus subtilis* HB0042 (ΔsigW) yielded two mutant strains, WY01 and RSpMarA2, respectively.

For mapping of transposon insertion sites, 5 μg of genomic DNA isolated from the transposant was digested with TaqI. The reaction mixture was circularized in a ligation reaction using a rapid ligation kit (Fermentas, Germany) at a DNA concentration of 5 ng/μl. Inverse PCR (IPCR) was performed with 100 ng of ligated DNA using primers oIPCR1 and oIPCR2.

---

**FIG 1** Identification of the *acn* operon by transposon mutagenesis. (A) Spot-on-lawn test onto *Bacillus subtilis* HB0042 (ΔsigW) with CH5 (Δsfp ΔyczE), which is devoid of Sfp-dependent nonribosomal synthesis of lipopeptides and polyketides, and WY01 (TnHimarI:RBAM_029230). (B) Bioassay with *Bacillus subtilis* HB0042 (ΔsigW). Fifty microliters of the supernatants of RS6 (Δsfp Δbac), which is devoid of nonribosomal synthesis of lipopeptides, polyketides, and bacilysin, RS16 (ΔRBAM_029190), RS17 (ΔRBAM_029230), RS18 (ΔRBAM_09240), and RS19 (ΔRBAM_029220) was applied. (C) The *acn* gene cluster of FZB42 flanked by two terminators (T) consists of six genes covering 4,490 bp. The operon is located between positions 3,048,678 and 3,044,445 on the genome of FZB42. (D) MALDI-TOF mass spectra of the culture supernatants of mutants RS6 and RS17 after precipitation with 80% ammonium sulfate. The pellet was extracted with methanol, and the extract was tested mass spectrometrically. In the mass spectrum of RS6, amylocyclicin was detected at m/z 6,382.2, while for mutant RS17, the bacteriocin was completely missing. Intens. [a.u.], intensity in absorbance units.
and oIPCR2, which face outwards from the transposon sequence. The IPCR products were purified using a PCR purification kit (Amersham, United Kingdom) and sequenced using primer oIPCR3.

**Bioassay for inhibitory activity.** LB agar (20 ml) was mixed with 0.5 ml of the indicator strain (OD at 600 nm [OD600] ~ 1.0). Twenty microliters of the culture supernatant was applied to a petri dish and incubated for 16 h at 22°C. Inhibitory activity appeared as a clear zone.

**Spot-on-lawn test.** Ten microliters of a liquid culture (OD600 ~ 4) of FZB42 and the mutant strains was spotted on a bioassay plate, and the plate was incubated for 16 h at 22°C. Antibacterial activity was observed as a clear zone around the spot (8).

**Mass spectrometric detection of amylocyclicin.** Amylocyclicin was detected in the culture supernatant by mass spectrometry (MS). For this purpose, 50 ml liquid production medium was inoculated into a 500-ml glass bulb with 500 ml of an LB overnight culture, shaken for about 6.5 h until the OD reached 8.5 (end of log phase), and centrifuged at 14,000 rpm for 5 min. Then, 50 ml supernatant was diluted with 200 ml distilled water and precipitated at 80% ammonium sulfate saturation. After centrifugation at 14,000 rpm for 5 min, the pellet was extracted with 250 μl methanol and centrifuged again. The supernatant was used for matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) mass spectrometric detection of amylocyclicin.

Surface extracts prepared by extraction of cell pellets with 50% acetone–0.1% trifluoroacetic acid were used for purification of amylocyclicin by high-pressure liquid chromatography (HPLC) and mass spectrometric characterization as well as proteolytic digestion and peptide sequencing of amylocyclicin.

**MALDI-TOF mass spectrometric analysis.** MALDI-TOF mass spectra were recorded using a Bruker Autoflex MALDI-TOF instrument containing a 337-nm nitrogen laser for desorption and ionization. Samples of 2 µl were mixed with the same volume of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile containing 0.1% [vol/vol] trifluoroacetic acid), spotted on the target, air dried, and measured as described previously (20). Spectra were obtained by positive ion detection and linear mode MS.

Proteolytic digestion of amylocyclicin was monitored with a Bruker Autoflex Speed MALDI-TOF/TOF mass spectrometer. The sample preparation method was the same as that outlined above. Sequencing of tryptic fragments was performed by MALDI LIFT-TOF/TOF mass spectrometry (21).

**Purification of amylocyclicin.** For proteolytic digestion and mass spectrometric sequencing, amylocyclicin was purified to homogeneity by reverse-phase HPLC (RP-HPLC) using an Agilent (1200 series) instrument. Surface extracts of the wild-type and mutant strains were evaporated to dryness in a SpeedVac evaporator. The dried material was dissolved in a minimum volume of 50% aqueous acetonitrile–0.1% trifluoroacetic acid. Extracts were applied to a Zorbax 300 SP-C8 column (4.6 by 150 mm; 3.5 μm; rapid solution). Eluent A was 0.1% trifluoroacetic acid in water; eluent B was 99.9% acetonitrile–0.1% trifluoroacetic acid. Amylocyclicin was eluted by a two-step gradient from 0 to 70% acetonitrile in water; eluent B was 99.9% acetonitrile–0.1% trifluoroacetic acid.

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**Proteolytic digestion and peptide fragment analysis.** Amylocyclicin was proteolytically digested by trypsin from bovine pancreas (Sigma-Aldrich, Deisenhofen, Germany). Lyophilized highly purified amylocyclicin obtained from a surface extract batch was dissolved in 60 µl 50% acetone–0.1% trifluoroacetic acid. For tryptic digestion of the bacteriocin, an aliquot of 15 µl was mixed with 15 µl 100 mM ammonium bicarbonate buffer, pH 8.2, 30 µl acetonitrile, and 10 µl H2O. Digestion was started by addition of 5 µl of a solution of trypsin in 1 mM HCl corresponding to 1 µg trypsin per test. Digestion was performed overnight. Fragmentation of amylocyclicin was controlled by MALDI-TOF MS at 0, 2, 4, 6, and 24 h.

As has been observed for similar compounds, amylocyclicin resists proteolytic digestion by endoproteases. In particular, amylocyclicin binds tightly to the surface of plastic vials. For this reason, it rapidly disappears from the digestion mixtures, with the consequence being that the bacteriocin cannot be proteolytically degraded. Therefore, it is essential that enzymatic digestion of amylocyclicin be performed in glass vials. Furthermore, addition of organic solvents, preferably, acetonitrile, at a concentration higher than 30% is necessary for successful fragmentation.

**Reconstruction of phylogenetic trees.** Peptide sequences were aligned by using the Clustal W program (22). A maximum likelihood matrix was calculated from this alignment by use of the PROML program. In order to assess the reliability of the trees, multiple data sets were generated with the SEQBOOT program. A tree was built from each replicate using PROML, and bootstrap values were computed with the TreeView (32-bit) program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Programs used to reconstruct the phylogenetic tree were obtained from the PHYLIP (v.3.69) software package (http://evolution.genetics.washington.edu/phylip).

**RESULTS**

**Detection of a gene cluster involved in bacteriocin biosynthesis.** It has been shown that the sfp mutant CH3, which is devoid of nonribosomal synthesis of antimicrobial secondary metabolites, produced at least one substance that was effective against *Bacillus subtilis* CU1065 and particularly against its sigW mutant, HB0042 (8). SigW is an extracytoplasmic sigma factor that provides intrinsic resistance to antimicrobial compounds produced by other bacilli (8). In order to detect the gene(s) responsible for the antibacterial activity, we prepared a *Himar1* mariner transposon library hosted in *sfp* mutant strain CH5 (Δsfp ΔyczE) following the procedure previously described by Le Breton et al. (19). A bioassay (see Materials and Methods) with about 2,000 mutant strains containing randomly distributed transposon insertions in their genomes was performed for mutants that had lost antibacterial activity against the *B. subtilis* sigW mutant HB00042. Two transposon mutants were selected (Fig. 1A). These mutant phenotypes were not due to an inactivation of the *bacilysin* (*bac*) gene cluster: mutant RS6, harboring knockout mutations in *sfp, yczE*, and *bac*, remained able to suppress the growth of HB00042 (Fig. 1B). Sequencing of the region next to the transposon insertion revealed that in mutant WY01, the *RBAM_029230* gene was interrupted by the transposon insertion, while mutant RSpMarA2 harbored an interrupted degU gene, suggesting that synthesis of the antibacterial compound is strictly dependent on DegU. DegU, a global transcription regulator, activates the nonribosomal synthesis of the polyketide difficidin, the antifungal lipopeptide bacillomycin D, and bacilysin in FZB42 (23, 24).

**Characterization of the gene cluster involved in the biosynthesis of a putative circular bacteriocin.** The gene cluster surrounding *RBAM_029230* was flanked by two terminators and comprised 4,490 bp with six open reading frames (Fig. 1C) following the protocol used for the *B. subtilis* sigW mutant HB00042. Two transposon mutants were selected (Fig. 1A). These mutant phenotypes were not due to an inactivation of the *bacilysin* (*bac*) gene cluster: mutant RS6, harboring knockout mutations in *sfp, yczE*, and *bac*, remained able to suppress the growth of HB00042 (Fig. 1B). Sequencing of the region next to the transposon insertion revealed that in mutant WY01, the *RBAM_029230* gene was interrupted by the transposon insertion, while mutant RSpMarA2 harbored an interrupted degU gene, suggesting that synthesis of the antibacterial compound is strictly dependent on DegU. DegU, a global transcription regulator, activates the nonribosomal synthesis of the polyketide difficidin, the antifungal lipopeptide bacillomycin D, and bacilysin in FZB42 (23, 24).

Characterization of the gene cluster involved in the biosynthesis of a putative circular bacteriocin. The gene cluster surrounding *RBAM_029230* was flanked by two terminators and comprised 4,490 bp with six open reading frames (Fig. 1C). Bioinformatic analysis revealed a structure similar to that in the gene clusters involved in the synthesis of known circular bacteriocins, e.g., enterocin AS48 (25), circularin A (26), carnocyclin (27), uboverolysin (28), butyrivibriocin (29), gasserinc (30), and lactocyclin (31). For this reason, it was assumed that the product of the gene cluster around *RBAM_029230* (in the following, named *acen*) might be involved in the synthesis and processing of a circular peptide which is the putative antibacterial substance acting against *Bacillus subtilis* HB00042.

To determine which genes within the *acen* gene cluster are essential for the biosynthesis of the antibacterial compound, each gene was individually replaced by a spectinomycin resistance gene cassette while maintaining the function of the downstream genes of the cluster. The results indicated that, besides *acen*, two other
members of the gene cluster (acnB and acnC) were essential for product formation, while a knockout mutation of the acnF gene did not affect the antagonistic action against HB00042 (Fig. 1B).

Genome mining using the whole acn FZB42 sequence revealed a high degree of sequence similarity to gene clusters present in some closely related representatives of the Bacillus subtilis group, including B. amyloliquefaciens subsp. plantarum UCMB5113, B. amyloliquefaciens subsp. amyloliquefaciens DSM7, B. subtilis subsp. subtilis RO-NN-1, and B. subtilis subsp. spinizenni W23, and also in the more distantly related species Bacillus coagulans and Paenibacillus larvae subsp. larvae (Table 2).

The first gene of the putative operon, acnB, encodes a membrane-anchored protein comprising five transmembrane helices with unknown function. AcnB homologues were not found in the clusters of known circular bacteriocins but were found in P. larvae subsp. larvae and B. coagulans. A weak homology (45%) to an ABC transporter from Lactobacillus ultonensis DSM16047 was found to exist, suggesting a possible function in export. The translated product of acnA (RBAM_029230) is a secretory protein with a deduced signal peptide (Table 2). The precursor protein consisted of 112 amino acids (aa). AcnA is similar to UblA (52% amino acid identity), the precursor of uberolysin. In both gene clusters, the peptide precursor gene was followed by a large gene encoding a membrane protein with a putative function in circularization and maturation of the bacteriocin precursor. AcnC, harboring 12 predicted transmembrane helices, displayed no homology to proteins involved in the formation of known circular bacteriocins. Because of structural similarity with the uberolysin cluster, AcnC could have the same function as UblB, a membrane protein with a putative maturation and circularization function, or the same function as AS-48B, which also contains 12 transmembrane helices. AcnC had weak homology to BacB of Enterococcus faecalis (27%). BacB is a plasmid-encoded immunity protein for the baca-encoded bacteriocin (32). It was suggested for AS-48 that the large membrane protein AS-48B alone or together with the export ABC transporter C4D forms a pore for the exit of the AS-48 bacteriocin (33). This could also be the case for AcnC and the downstream ABC transporter proteins AcnD and AcnE.

AcnD is the ATP-binding protein of the ABC transporter, while AcnE is an integral membrane protein with four putative transmembrane helices belonging to the DUF95 superfamily. AcnD and AcnE displayed structural similarity to the proteins occurring in gene clusters for known circular bacteriocins. We did not obtain knockout mutants for acnD and acnE, suggesting that their products might play an important role in the self-immunity of FZB42 against amylolycin.

AcnF, with its small size, its high charge, and its membrane association due to three predicted transmembrane helices, is similar to the typical immunity peptides of circular bacteriocins (33). As expected, the acnF mutant strain RS16 still suppressed the growth of HB00042 (Fig. 1B).

Identification of the acnA gene product and formation of the mature amylolycin. On the basis of a comparison of the sequence of the amylolycin precursor from FZB42 with that of uberolysin, the molecular mass of the acnA product without a leader peptide (64 amino acids) was calculated to be m/z 6,399.6. The high pI of 9.82 is in accordance with the pI of other circular bacteriocins (27). Given a head-to-tail cyclization, a molecular mass of 6,381.6 Da (6,399.6 Da with H2O) was predicted for the processed acnA gene product.

Production of this predicted product was investigated by the use of MALDI-TOF MS measurements for FZB42 and mutant strains. The mass spectra of methanolic extracts of ammonium sulfate precipitates from culture supernatants and surface extracts of the wild-type strain FZB42 and mutant strain RS6 showed a compound with a mass of 6,382 Da for the [M + H]+ species of the mature AcnA peptide. As expected, this peak was not detected in samples from the mutant RS17 harboring a knockout mutation in the acnA gene (Fig. 1D). Note that this product is 18 mass units lower than the mass of 6,400 Da predicted for the mature AcnA product, as calculated from the gene sequence. The molecular mass of 6,382 Da for [M + H]+ species is consistent with the processing of the prepeptide by elimination of 48 out of the 112 amino acids that belong to the leader peptide at the N terminus, followed by circularization of the 64 amino acids comprising bacteriocin by a peptide bond between the N-terminal leucine and the tryptophan at the C terminus, which is a typical feature of circular bacteriocins.

Due to the peptide character, its cyclic structure, and the fact that several Bacillus amyloliquefaciens strains carry this gene clus-

### Table 2: Characterization of acn gene cluster of FZB42

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<th>Gene Cluster</th>
<th>Protein</th>
<th>No. of amino acids</th>
<th>pI</th>
<th>TMH</th>
<th>SP</th>
<th>Homology to P. larvae and B. coagulans</th>
<th>Putative function</th>
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<td>RBAM_029240</td>
<td>AcnB</td>
<td>190</td>
<td>9.39</td>
<td>5</td>
<td>0.784</td>
<td>WP_023484874, 46%; YP_004860046, 36%</td>
<td>Transmembrane protein, essential for synthesis</td>
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<td>RBAM_029230</td>
<td>AcnA</td>
<td>112</td>
<td>9.75</td>
<td>3</td>
<td>1.000</td>
<td>WP_023484873, 80%; YP_004860047, 63%</td>
<td>Precursor of circular bacteriocin, essential for synthesis</td>
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<td>RBAM_029220</td>
<td>AcnC</td>
<td>551</td>
<td>9.66</td>
<td>12</td>
<td>0</td>
<td>WP_023484872, 52%; YP_004860048, 37%</td>
<td>Processing, maturation, and self-immunity; essential for synthesis</td>
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<tr>
<td>RBAM_029210</td>
<td>AcnD</td>
<td>234</td>
<td>5.76</td>
<td>0</td>
<td>0</td>
<td>WP_023484871, 74%; YP_004860049, 55%</td>
<td>ABC transporter, ATP-binding, self-immunity</td>
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<td>RBAM_029200</td>
<td>AcnE</td>
<td>149</td>
<td>7.76</td>
<td>3</td>
<td>0</td>
<td>WP_023484870, 54%; YP_004860050, 45%</td>
<td>Integral membrane protein, DUF95 superfamily, self-immunity</td>
</tr>
<tr>
<td>RBAM_029190</td>
<td>AcnF</td>
<td>73</td>
<td>9.70</td>
<td>3</td>
<td>&lt;0.1</td>
<td>Not present in P. larvae subsp. larvae; YP_004860051, 47%</td>
<td>Transmembrane protein, nonessential for synthesis</td>
</tr>
</tbody>
</table>

* TMH, number of transmembrane helices predicted by the TMHMM server, v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/).
* SP, probability of a signal sequence predicted by the SignalP program, v.3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/).
* Homology (percentage of identical amino acid residues) to genes present in unknown gene clusters of Paenibacillus larvae subsp. larvae (sequences with GenBank accession numbers WP_023484870 to WP_023484874) and Bacillus coagulans subsp. larvae (sequences with GenBank accession numbers YP_004860046 to YP_004860051).
ter, we named this antibacterial peptide with similarity to the circular bacteriocin uberolysin amylocyclicin (Acn). In addition to RS17 (acnA), amylocyclicin was not detected in mutant strain RS18 (acnB) or RS19 (acnC) or in mutant strain WY06 carrying a complete deletion of the acn gene cluster (see Fig. S1 in the supplemental material).

**Purification and chemical characterization of amylocyclicin.**
Amylocyclicin is released into the culture medium by wild-type strain *B. amyloliquefaciens* FZB42 and sfp mutants derived therefrom. It can be obtained from ammonium sulfate precipitation of the supernatants, followed by extraction of the pellet with methanol. In addition, the bacteriocin is attached in an appreciable amount to the outer surface of the bacterial cells, from where it can be extracted with 50% aqueous acetonitrile–0.1% trifluoroacetic acid. Such surface extracts are the source of choice for further purification and characterization of the bacteriocin. Using this material, amylocyclicin was purified to homogeneity in one step by RP-HPLC using a Zorbax 300 SB-C8 column. A MALDI-TOF mass spectrum of the surface extract of mutant RS6 (H9004 sfp H9004 yczE H9004 bac) is shown in Fig. 2A. Amylocyclicin, found at m/z 6,381.4, is a major component in this extract. The mass spectrum of the pure bacteriocin obtained by HPLC is shown in Fig. 2B. The mass for the [M + H]⁺ protonated form of amylocyclicin was found at m/z 6,381.4,
while the mass peak at \( m/z \) 3,190.3 was attributed to the doubly charged form.

Amylocyclicin is a highly hydrophobic cyclic peptide with a molecular mass of 6,381 Da, as determined by MALDI-TOF MS. Its high pI of 9.82 is in accordance with the pIs of other circular bacteriocins. In order to identify the site of cyclization in the peptide ring of amylocyclicin, tryptic digestion in combination with mass spectrometric sequence analysis was performed. Six tryptic peptide fragments that were obtained are listed in Fig. 3, and these fragments include the putative W-L cyclization site. Peptide sequencing was performed using MALDI LIFT-TOF/TOF mass spectrometry (21). For example, sequence analysis is demonstrated in Fig. 4 for the tryptic fragment found at \( m/z \) 1,766.2. Here, a complete set of \((Y_n + H_2O)\) ions and numerous \(b_n\) ions were obtained, which allowed sequence determination. In this way, the hypothetical cyclization of amylocyclicin by peptide bond formation between the N-terminal leucine and the tryptophan at the C terminus was verified.

**Self-immunity against amylocyclicin is governed by AcnC, AcnD, AcnE, and AcnF.** To investigate which of the genes of the \(\text{acnA}\) cluster are involved in self-immunity, gene insertion mutants of the \(\text{acn}\) cluster were used as indicator strains in a spot-on-lawn bioassay (Fig. 5A). Generally, immunity proteins of circular bacteriocins are small cationic peptides, like AcnF, that often work in cooperation with an ABC transporter to give full immunity (15). The \(\text{acnF}\) insertion mutant RS16 was found, surprisingly, to have sensitivity to amylocyclicin (RS6), excluding the possibility of an important function of AcnF in self-immunity, while the \(\text{acnC}\) insertion mutant RS19 was clearly more sensitive. We inferred that the unknown product of \(\text{acnC}\) was important both in self-immunity against amylocyclicin and in amylocyclicin synthesis. Complete deletion of the cluster in the mutant WY06 resulted

![FIG 3](image-url)  Processing and circularization of amylocyclicin. The N-terminal leader peptide comprising 48 amino acids is eliminated by cleavage of the peptide bond between E\(^{-1}\) and L\(^1\) (vertical arrow), followed by formation of a new peptide bond between W\(^{64}\) and L\(^1\), yielding the circular bacteriocin. Six tryptic fragments were detected by MALDI-TOF mass spectrometry and included the cyclization site W-L, which is indicated by bold letters. Mass spectrometric peptide sequencing was performed using a Bruker AutoFlex TOF TOF instrument and the LIFT technique (36).

![FIG 4](image-url)  Mass spectrometric sequencing of the tryptic fragment of amylocyclicin detected at \( m/z \) 1,766.2 using the MALDI LIFT-TOF/TOF MS technique. The sequence was derived from a complete set of \((Y_n + H_2O)\) ions and numerous \(b_n\) ions. In this way, the predicted circularization of amylocyclicin between L\(^1\) and W\(^{64}\) was verified.
in a dramatic increase in sensitivity to amylocyclicin. This was presumably due to the simultaneous absence of AcnF and AcnC and to the absence of the ABC transporter proteins AcnD and AcnE. In addition, we could not get mutants of the ABC transporters AcnD and AcnE, underlining their major role in immunity, possibly due to their function in the export of amylocyclicin.

Transcription factor SigW is involved in general immunity against amylocyclicin and other substances produced by *B. amyloliquefaciens* FZB42. Immunity against amylocyclicin was also found to be dependent on SigW, a global transcription regulator involved in general immunity against different bacteriocins (8). *Bacillus subtilis* 168 and its phase mutant, CU1065 (attSPB), were sensitive to the amylocyclicin producer strain RS6, while the nonproducer strain RS17 was much less efficient in cell killing (Fig. 5B). Remarkably, the *Bacillus subtilis* sigW mutants HB10102 and HB0042 were more sensitive to amylocyclicin than their wild-type counterparts. The *acn* mutant RS17 had reduced activity against these sigW mutants, indicating that SigW provides resistance against amylocyclicin in *Bacillus subtilis*.

We cannot rule out the possibility that bacteriocins other than amylocyclicin were ribosomally synthesized by FZB42. As shown in Fig. 5B, the *acn* insertion mutant RS17 was unable to kill *B. subtilis* 168 and CU1065, while activity against the corresponding sigW mutants was reduced, but not abolished. The other ribosomally synthesized compound produced in FZB42, plantazolicin (9), was not involved in this activity, since the plantazolicin overproducer RSpMarA2 was unable to inhibit *B. subtilis* (data not shown). Which metabolite is responsible for this activity remains unknown, but its synthesis seems to be dependent on DegU, like the synthesis of amylocyclicin does.

The SigW-dependent operon *ydbST* governs the immunity of *Bacillus subtilis* to amylocyclicin. The SigW-dependent *ydbST* operon is involved in the general immunity of *B. subtilis* against bacteriocins produced by *B. amyloliquefaciens* FZB42. The operon is well conserved in the *B. subtilis* species complex, including FZB42. Homologues of the *ydbST* genes have been identified in a *Staphylococcus aureus* plasmid encoding the production of and immunity to the bacteriocin aureocin A53. Genetic analysis suggests that at least YdbT functioned in aureocin immunity (34).

We found that the *ydbST* mutant strain HB6213 was nearly as sensitive as the sigW mutant strains HB10102 and HB0042 to the amylocyclicin producer strain RS6 (Fig. 5C).

Another gene involved in the immunity of *B. subtilis* against bacteriocins is the bacillithiol-dependent thiol transferase FosB (35). The mechanism by which this might confer resistance is not clear, but perhaps there are free Cys thiols that could be coupled to bacillithiol or an electrophilic site that could be targeted by the thiol transferase function.

We performed spot-on-lawn tests with the *Bacillus subtilis* fosB mutant strain HB0008 and the double mutant HB6131 (Δ*ydbST* Δ*fosB*). Both *ydbST* and *fosB* were found to be involved in the general immunity of *B. subtilis* against amylocyclicin (Fig. 5C).

**Amylocyclicin inhibits Gram-positive but not Gram-negative bacteria**. Amylocyclicin, a representative of circular bacteriocins, was identified to be a antibacterial substance acting against *Bacillus subtilis*. Therefore, we investigated the antibacterial effects of amylocyclicin against some representatives of Gram-positive and Gram-negative bacteria. Activity tests performed with the culture supernatants and spot-on-lawn tests with strain RS6 and the amylocyclicin mutant strain RS17 revealed a high level of activity of amylocyclicin against Gram-positive bacteria but not against Gram-negative bacteria, such as *E. coli* and *Erwinia* spp. (Table 3; see Fig. S2 in the supplemental material). These findings were in line with the results obtained with other circular bacteriocins produced by Gram-positive bacteria (33).

**DISCUSSION**

A circular, highly hydrophobic peptide named amylocyclicin with a molecular mass of 6,381 Da was identified as the compound responsible for the reported activity of *B. amyloliquefaciens* FZB42.
against *B. subtilis* HB0042. In addition, the gene cluster responsible for the synthesis, posttranslational modification, and self-immunity (*acn*) of amylocyclicin was identified in *B. amyloliquefaciens* subsp. *plantarum* FZB42. The second gene of the cluster, *acnA*, encodes a linear precursor peptide consisting of 112 aa. After posttranslational removal of an exceptionally large N-terminal leader peptide (48 aa), the mature peptide consists of 64 aa. The site of cyclization between the N-terminal Leu and the C-terminal Trp was corroborated by peptide sequencing using MALDI LIFT-TOF/TOF mass spectrometry. Amylocyclicin is a member of the circular bacteriocins, a growing family of ribosomally synthesized peptides with a posttranslational head-to-tail cyclization of their backbone. They are distinguished from other bacteriocins by their thermostability and high pI values. Most circular bacteriocins probably adopt a common three-dimensional structure consisting of several α helices encompassing a hydrophobic core (15). They have been detected in numerous Gram-positive bacteria and were found to act against closely related bacteria by causing nonselective pores in their membranes (36). The mature amylocyclicin displayed only weak sequence similarity to known representatives of circular bacteriocins: uberolysin from *Streptococcus uberis* (28) (39.1% identity), leucocyclicin Q from *Leuconostoc mesenteroides* (37) (37.5%), carnocyclin A from *Carnobacterium maltaromaticum* (27) (34.4%), garvicin from *Lactobacillus gasseri* (30) (21.9%), and acidocin B from *Lactobacillus acidophilus* (40) (21.9%). Together with translated sequences found in other representatives of the *B. subtilis* group (97 to 98% identity), *Paenibacillus larvae* subsp. *larvae* (98%), and *Bacillus coagulans* (94%), amylocyclicin forms a separate cluster, which is distantly related to the *pznA*-like sequences present in the genomes of *Staphylococcus aureus* (70% identity), *Sporolactobacillus vineae* (67%), *Marinitoga piezophila* (64%), *Enterococcus* spp. (61%), and *Clostridium perfringens* (50%). Sequences in several *Streptococcus* strains and the presently

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**TABLE 3 Activity spectrum of amylocyclicin**

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>Inhibitiona</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus brevis</em> ATCC 8246b</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> ATCC 14579</td>
<td>+</td>
</tr>
<tr>
<td><em>Clavibacter michiganensis</em> NCPPB382</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em> ATCC 9789</td>
<td>+</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus pumilus</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> CU1065</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> HB0042</td>
<td>+ +</td>
</tr>
<tr>
<td><em>Bacillus sphaericus</em> ATCC 14577</td>
<td>+ +</td>
</tr>
<tr>
<td><em>Paenibacillus polymyxa</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Paenibacillus granivorans</em></td>
<td>+ +</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em> 7A1</td>
<td>–</td>
</tr>
<tr>
<td><em>Arthrobacter</em> sp.</td>
<td>–</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>–</td>
</tr>
<tr>
<td><em>E. coli</em> K-12</td>
<td>–</td>
</tr>
<tr>
<td><em>Klebsiella terrigena</em></td>
<td>–</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>–</td>
</tr>
<tr>
<td><em>Erwinia carotovora</em></td>
<td>–</td>
</tr>
</tbody>
</table>

*ATCC, American Type Culture Collection.*  
*a Degree of inhibition in a bioassay: ++, strong inhibition; +, inhibition; −, no inhibition.*

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**FIG 6** Maximum likelihood phylogenetic tree of mature circular bacteriocins using subtilosin A as an outgroup. The consensus tree was reconstructed from 100 trees according to the extended majority rule (SEQBOOT program). Bootstrap values of ≥50% are indicated at branch points. The species and GenBank accession numbers are indicated. References for the bacteriocins are given in the text. Bar, 0.1 substitution per amino acid position.
known circularins form disparate clusters in the phylogenetic trees constructed by the maximum likelihood (Fig. 6) or neighbor joining (see Fig. S3 in the supplemental material) programs of the PHYLIP package.

Our results demonstrate that immunity against amylocyclicin in the producer strain FZB42 and in closely related strains also belonging to the B. subtilis group is governed by two different mechanisms: (i) self-immunity governed by gene products encoded by several acn genes, some of which are probably involved in export through the cytoplasmic membrane, and (ii) general immunity in B. subtilis against bacteriocins, including amylocyclicin and other antibacterial compounds, governed by gene products whose synthesis is sigma W dependent. This suggests that several mechanisms of protecting against antibiotic stress which are caused by circular bacteriocins have evolved in the competitive environment of the plant rhizosphere and in other ecological niches.

Besides subtilosin A (41), amylocyclicin is the first representative of circular bacteriocins found in the genus Bacillus. However, the subtilosin secreted by B. subtilis differs from regular bacteriocins. Subtilosin is now considered the prototype of its own (sub)class of bacteriocins known as sactibiotics, since it is extensively posttranslationally modified by three covalent thioether bonds, besides the linkage between the N and C termini and cleavage of the N-terminal extension (42). Gene clusters mirroring the acn gene cluster in FZB42 were detected in Bacillus subtilis subsp. subtilis RO-NN-1, Bacillus subtilis subsp. spizizenii W23, Bacillus coagulans 36D1, and Paenibacillus larvae subsp. larvae. Therefore, we assume that several representatives of the Bacillus/Paenibacillus taxon produce amylocyclicin-like bacteriocins.

ACKNOWLEDGMENTS

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REFERENCES


