Amylocyclicin, a Novel Circular Bacteriocin Produced by *Bacillus amyloliquefaciens* FZB42

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Bacillus amyloliquefaciens FZB42 is a Gram-positive plant growth-promoting bacterium with an impressive capacity to synthesize nonribosomal secondary metabolites with antimicrobial activity. Here we report on a novel circular bacteriocin which is ribosomally synthesized by FZB42. The compound displayed high antibacterial activity against closely related Gram-positive bacteria. Transposon mutagenesis and subsequent site-specific mutagenesis combined with matrix-assisted laser desorption ionization–time of flight mass spectroscopy revealed that a cluster of six genes covering 4,490 bp was responsible for the production, modification, and export of and immunity to an 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 let us assume that this type of peptide is widespread among the *Bacillus/Paenibacillus* taxon.

Bacillus amyloliquefaciens FZB42 is a Gram-positive soil bacterium that promotes plant growth and has a huge potential to produce nonribosomal secondary metabolites (1). These are the 4'-phosphopantetheine transferase (Sfp)-dependent polyketides bacillaene, diffcadin, and macrolactin (2, 3), the lipopeptides surfactin, fengycin, and bacillomycin D (4), the siderophore bacillibactin, and the putative product of the *nrs* cluster (5). Nonribosomal synthesis of the antibacterial dipeptide bacilysin is independent of Sfp (6). Bacilysin and the polyketides, especially diffcadin, possess strong antagonistic activity against Gram-positive and Gram-negative bacteria (2, 6). In total, 8.5% of the *B. amyloliquefaciens* FZB42 genomic capacity is devoted to nonribosomal synthesis of secondary metabolites, a capacity that exceeds that of the closely related model Gram-positive bacterium *Bacillus subtilis* 168 by more than 2-fold (7). Biosynthetic gene clusters of ribosomally synthesized peptide antibiotics, which are common in *B. subtilis* strains, until recently remained unknown in FZB42, despite the finding of a substance produced by the FZB42 mutant strain CH3, devoid of nonribosomal synthesis of secondary metabolites, that acts against *Bacillus subtilis* and especially against its *sigW* mutant, HB0042 (8).

The striking sensitivity of the *B. subtilis* *sigW* mutant to strain FZB42 motivated us to search for ribosomally produced antibacterial substances. Recently, we detected a strongly modified peptide, plantazolicin, produced by a *B. amyloliquefaciens* FZB42 mutant strain deficient in nonribosomal synthesis (9). The compound belongs to the TOMM group of thiazole/oxazole-modified microcins (10, 11), and its structure has been elucidated to contain a hitherto unusual number of thiazoles and oxazoles formed from a linear 14-mer precursor peptide (12). It acts against closely related bacilli, especially *B. anthracis* (13). Furthermore, plantazolicin possesses nematocidal activity (14), but it is not responsible for the reported strong activity against the *Bacillus subtilis* *sigW* mutant HB0042 (8).

Here, we identify a gene cluster responsible for ribosomal syn-

thesis of a bacteriocin peptide with similarity to circular nonantibiotic bacteriocins produced by lactic acid bacteria and other Gram-positive bacteria (15). Peptide sequencing of the fragments obtained after tryptic digestion of the purified peptide corroborated circularization of the novel compound.

MATERIALS AND METHODS

Cultivation of FZB42 and mutant strains for molecular biological studies. The *B. amyloliquefaciens* 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 soy peptone, 40 g dextrin 10, 1.8 g KH₂PO₄, 4.5 g K₂HPO₄, 0.3 g MgSO₄ · 7H₂O, and 0.2 ml KellyT trace metal solution per liter was used. KellyT trace metal solution consists of 25 mg EDTA disodium salt dihydrate, 0.5 g ZnSO₄ · 7H₂O, 3.67 g CaCl₂ · 2H₂O, 1.25 g MnCl₂ · 4H₂O, 0.25 g CoCl₂ · 6H₂O, 0.25 g ammonium molybdate, 2.5 g FeSO₄ · 7H₂O, and 0.1 g CuSO₄ · 5H₂O in 500 ml 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. amyloliquefaciens* FZB42 and mutant strain RS6 were grown in half-

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>Bacillus subtilis</i>		
CU1065	168 <i>trpC2 attSPβ</i>	2
HB0042	168 <i>trpC2 attSPβ sigW::kan</i>	2
HB10102	168 <i>sigW::mIs</i>	2
HB0008	CU1065 <i>fosB::Cm</i>	2
HB101031	CU1065 <i>ydbST::kan fosB::Cm</i>	2
HB101013	CU1065 <i>ydbST::kan</i>	2
<i>Bacillus megaterium</i> 7A/1	Indicator strain for polyketides	Laboratory stock
<i>Bacillus amyloliquefaciens</i>		
FZB42	Type strain <i>B. amyloliquefaciens</i> subsp. <i>plantarum</i>	DSMZ (DSMZ23117), BGSC (10A6)
CH5	FZB42 <i>sfp::ermAM yczE::Cm</i>	6
RSpMarA2	Insertion of pMarA in CH5 <i>degU::kan</i>	This work
RS6	<i>sfp::ermAM bac::Cm</i> , deficient in lipopeptides, polyketides, and bacilysin	6
RS16	RS6 <i>ΔacnF::spc</i>	This work
RS17	RS6 <i>ΔacnA::spc</i>	This work
RS18	RS6 <i>ΔacnB::spc</i>	This work
RS19	RS6 <i>ΔacnC::spc</i>	This work
WY06	CH5 <i>ΔacnBACDEF</i>	This work
WY01	CH5 transposon mutant <i>RBAM_029230::kan</i>	This work
Plasmids		
pGEM-T	<i>Ap^r lacZ</i>	Promega
pMarA	Plasmid containing mariner transposon TnYLB-1	19
pIC333	Plasmid with spectinomycin resistance cassette	T. Msadek, Institute Pasteur, Paris, France
pGEM-T _{km}	pGEM-T containing kanamycin resistance cassette	This work
pGEM-T _{kmR}	pGEM-T _{km} with right flanking site of <i>acn</i> cluster	This work
pGEM-T _{kmRL}	pGEM-T _{kmR} with right and left flanking sites of <i>acn</i> cluster	This work

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 1.0×10^3 CFU/ml and shaken at 120 rpm and 30°C. The culture was harvested after 24 h and centrifuged at 4,000 rpm and 4°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. 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. Spectinomycin (90 μg/ml) was used for the selection of transformants.

Deletion of the whole *acn* gene cluster, yielding mutant WY06, 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 flanking 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 flanking the left side of the *acn* gene cluster was amplified with

the primers Guac-N-1-sphI and Guac-N-2-sacII and then inserted between the SacII and SphI restriction sites of pGEM-T_{kmR} to create pGEM-T_{kmRL}.

To avoid polar effects, the mutants RS16, RS17, RS18, and RS19 were generated by gene splicing using the SOE method (18). This method assists with avoiding possible polar effects caused by interrupted reading frames. SOE PCR fusion products were generated using the primers listed in Table S1 in the supplemental material, the spectinomycin resistance gene of pIC333, and chromosomal DNA of *Bacillus amyloliquefaciens* FZB42. The PCR product was used for transformation after purification using a NucleoSpin Extract II kit from Macherey-Nagel (Düren, Germany).

Mutants without inhibitory activity against a *B. subtilis* *sigW* mutant strain were isolated from a mariner transposon pMarA library prepared in strain CH5 (*Δsfp ΔyczE*) as described by Le Breton et al. (19). In brief, strain CH5 (*Δsfp ΔyczE*) was transformed with plasmids pMarA, pMarB, and pMarC. Competent cells were obtained by modifying the two-step protocol of Kunst and Rapoport (16) as described by Idris et al. (17). Transformants were screened for plasmid-associated properties, i.e., Kan^r and Erm^r at the permissive temperature for plasmid replication (30°C) and Kan^r and Erm^s 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.

FIG 1 Identification of the *acn* operon by transposon mutagenesis. (A) Spot-on-lawn test onto *Bacillus subtilis* HB0042 ($\Delta sigW$) with CH5 ($\Delta sfp \Delta yczE$), which is devoid of Sfp-dependent nonribosomal synthesis of lipopeptides and polyketides, and WY01 (Tn*HimarI*::*RBAM_029230*). (B) Bioassay with *Bacillus subtilis* HB0042 ($\Delta sigW$). Fifty microliters of the supernatants of RS6 ($\Delta sfp \Delta bac$), which is devoid of nonribosomal synthesis of lipopeptides, polyketides, and bacilysin, RS16 ($\Delta RBAM_029190$), RS17 ($\Delta RBAM_029230$), RS18 ($\Delta RBAM_09240$), and RS19 ($\Delta RBAM_029220$) 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.

Selection for the loss of antibacterial activity using a bioassay (see below) with *Bacillus subtilis* HB0042 as the indicator strain yielded two mutant strains, WY01 and RSpMarA2, respectively.

For mapping of transposon insertion sites, 5 μ g 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/ μ l. Inverse PCR (IPCR) was performed with 100 ng of ligated DNA using primers oIPCR1

and oIPCR2, which face outwards from the transposon sequence. The PCR 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 amylocyclin. Amylocyclin 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 µl 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 amylocyclin.

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

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 amylocyclin 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 amylocyclin. For proteolytic digestion and mass spectrometric sequencing, amylocyclin 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. Amylocyclin was eluted by a two-step gradient from 0 to 70% eluent B in 70 min and from 70 to 95% eluent B in 5 min (70 to 75 min), followed by isocratic elution at 95% eluent B for 10 min.

Proteolytic digestion and peptide fragment analysis. Amylocyclin was proteolytically digested by trypsin from bovine pancreas (Sigma-Aldrich, Deisenhofen, Germany). Lyophilized highly purified amylocyclin obtained from a surface extract batch was dissolved in 60 µl 50% acetonitrile–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 amylocyclin was controlled by MALDI-TOF MS at 0, 2, 4, 6, and 24 h.

As has been observed for similar compounds, amylocyclin resists proteolytic digestion by endoproteases. In particular, amylocyclin 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 amylocyclin 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 *HimarI* mariner transposon library hosted in *sfp* mutant strain CH5 ($\Delta sfp \Delta yczE$) 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 bacillo-mycin 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), uberolysin (28), butyriovibriocin (29), gassericin (30), and lactocyclin (31). For this reason, it was assumed that the product of the gene cluster around *RBAM_029230* (in the following, named *acn*) 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 *acn* 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 *acnA*, two other

TABLE 2 Characterization of *acn* gene cluster of FZB42

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

^a TMH, number of transmembrane helices predicted by the TMHMM server, v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>).

^b SP, probability of a signal sequence predicted by the Signal P program, v.3.0 (<http://www.cbs.dtu.dk/services/SignalP-3.0/>).

^c 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* (sequences with GenBank accession numbers YP_004860046 to YP_004860051).

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. *spizizenii* 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 ultunensis* 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 C₁D 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 amylocyclin.

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 amylocyclin. On the basis of a comparison of the sequence of the amylocyclin 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 pIs 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 cluster,

FIG 2 Preparation of amylocyclin in pure form. (A) MALDI-TOF mass spectrum of a surface extract of mutant RS6. Amylocyclin, which is a major component, was detected at m/z 6,381.5. (B) Purification of amylocyclin by RP-HPLC. The lyophilized surface extract was dissolved in a minimum volume of 50% acetonitrile–0.1 trifluoroacetic acid and applied to a Zorbax 300 SP-C8 column. Amylocyclin was eluted by an acetonitrile gradient in pure form, as described in Materials and Methods. The mass spectrum of the purified bacteriocin shows peaks at m/z 6,381.4 and 3,190.3 which correspond to the $[M + H]^+$ and the doubly charged species, respectively.

ter, we named this antibacterial peptide with similarity to the circular bacteriocin uberolysin amylocyclin (Acn). In addition to RS17 (*acnA*), amylocyclin 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 amylocyclin.

Amylocyclin 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, amylocyclin 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 ($\Delta sfp \Delta yczE \Delta bac$) is shown in Fig. 2A. Amylocyclin, found at m/z 6,381.5, 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 amylocyclin was found at m/z 6,381.4,

Amylocyclin 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 amylocyclin, 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 amylocyclin by peptide

Self-immunity against amylocyclicin is governed by AcnC, AcnD, AcnE, and AcnF. To investigate which of the genes of the *acnA* cluster are involved in self-immunity, gene insertion mutants of the *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 *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 *acnC* insertion mutant RS19 was clearly more sensitive. We inferred that the unknown product of *acnC* was important both in self-immunity against amylocyclicin and in amylocyclicin synthesis. Complete deletion of the cluster in the mutant WY06 resulted

FIG 5 Spot-on-lawn tests for detecting immunity against the amylocyclin producer strain RS6. (A) Self-immunity against amylocyclin in FZB42 is directed by the *acn* genes. *B. amyloliquefaciens* strains RS6, WY06 ($\Delta acnBACDEF$), RS19 ($\Delta acnC$), and RS16 ($\Delta acnF$) were added to 1.5% LB agar at low density and poured as a lawn. The amylocyclin producer RS6 and the nonproducer RS17 ($\Delta acnA$) were spotted on the lawns at a high density. A zone of clearing is observed if the lawn strain is sensitive to the amylocyclin produced by RS6. No growth inhibition was detected when the amylocyclin nonproducer strain RS17 was spotted on the lawns. Lawn strains with increased sensitivity were RS16 ($\Delta acnF$), RS19 ($\Delta acnC$), and WY06 ($\Delta acnBACDEF$), while RS6 containing the intact *acn* gene cluster was found to be immune against amylocyclin. (B) SigW is necessary for the general immunity of *Bacillus subtilis* (*B. s.*) against amylocyclin and unknown compounds produced by RS17. Amylocyclin producer (RS6) and nonproducer (RS17) strains were spotted onto lawns formed by *B. subtilis* strains 168 (*trpC2*) and CU1065 (*attSPB*) and *sigW* mutant strains HB10102 and HB0042. The sensitivity of the *sigW* mutant strains to amylocyclin was enhanced. In addition, the growth of the *B. subtilis* *sigW* mutant strains was inhibited when the amylocyclin nonproducer strain RS17 was spotted, indicating that the *sigW* mutant strains became increasingly sensitive to an unknown compound still produced in RS17. (C) General immunity against amylocyclin in *B. subtilis* is provided by the SigW-dependent *ydbST* and *fosB* genes. Spot-on-lawn tests with *Bacillus subtilis* mutants HB6213 (*ydbST::kan*), HB0008 (*fosB::Cm*), and HB6131 (*ydbST::kan fosB::Cm*) against amylocyclin producer RS6 revealed enhanced sensitivity, while the growth inhibition effect of the *acnA* mutant RS17 was marginal.

in a dramatic increase in sensitivity to amylocyclin. 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 amylocyclin.

Transcription factor SigW is involved in general immunity against amylocyclin and other substances produced by *B. amyloliquefaciens* FZB42 in *Bacillus subtilis*. Immunity against amylocyclin 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 phage mutant, CU1065 (*attSPB*), were sensitive to the amylocyclin 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 amylocyclin than their wild-type counterparts. The *acn* mutant RS17 had reduced activity against these *sigW* mutants, indicating that SigW provides resistance against amylocyclin in *Bacillus subtilis*.

We cannot rule out the possibility that bacteriocins other than amylocyclin 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 amylocyclin does.

The SigW-dependent operon *ydbST* governs the immunity of *Bacillus subtilis* to amylocyclin. 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 amylocyclin 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 ($\Delta ydbST \Delta fosB$). Both *ydbST* and *fosB* were found to be involved in the general immunity of *B. subtilis* against amylocyclin (Fig. 5C).

Amylocyclin inhibits Gram-positive but not Gram-negative bacteria. Amylocyclin, a representative of circular bacteriocins, was identified to be a antibacterial substance acting against *Bacillus subtilis*. Therefore, we investigated the antibacterial effects of amylocyclin 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 amylocyclin mutant strain RS17 revealed a high level of activity of amylocyclin 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 amylocyclin with a molecular mass of 6,381 Da was identified as the compound responsible for the reported activity of *B. amyloliquefaciens* FZB42

TABLE 3 Activity spectrum of amylocyclicin

Indicator strain	Inhibition ^a
<i>Bacillus brevis</i> ATCC 8246 ^b	+
<i>Bacillus subtilis</i> 168	+
<i>Bacillus cereus</i> ATCC 14579	+
<i>Clavibacter michiganensis</i> NCPPB382	++
<i>Bacillus licheniformis</i> ATCC 9789	+
<i>Micrococcus luteus</i>	++
<i>Bacillus pumilus</i>	+
<i>Bacillus subtilis</i> CU1065	+
<i>Bacillus subtilis</i> HB0042	++
<i>Bacillus sphaericus</i> ATCC 14577	++
<i>Paenibacillus polymyxa</i>	+
<i>Paenibacillus granivorans</i>	++
<i>Bacillus megaterium</i> 7A1	+
<i>Arthrobacter</i> sp.	–
<i>Staphylococcus aureus</i>	–
<i>E. coli</i> K-12	–
<i>Klebsiella terrigena</i>	–
<i>Pseudomonas</i> sp.	–
<i>Erwinia carotovora</i>	–

^a Degree of inhibition in a bioassay: ++, strong inhibition; +, inhibition; –, no inhibition.

^b ATCC, American Type Culture Collection.

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 *Lactococcus garvieae* (38) (34.4%), enterocin AS-48 from *Enterococcus faecalis* (39) (34.4%), lactocyclicin Q from a *Lactococcus* sp. (37) (32.8%), circularin A from *Clostridium beijerinckii* (26) (31.3%), butyriovibriocin AR10 from *Butyriovibrio fibrisolvens* (29) (23.4%), gasserin A 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

FIG 6 Maximum likelihood phylogenetic tree of mature circular bacteriocins using subtilisin A as an outgroup. The consensus tree was reconstructed from 100 trees according to the extended majority rule (SEQBOOT program). Bootstrap values of $\geq 50\%$ 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 amylocyclin 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 amylocyclin 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 subtilisin A (41), amylocyclin is the first representative of circular bacteriocins found in the genus *Bacillus*. However, the subtilisin secreted by *B. subtilis* differs from regular bacteriocins. Subtilisin 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 amylocyclin-like bacteriocins.

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