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Engineered Biosynthesis of the Peptide Antibiotic Bacitracin in the Surrogate Host *Bacillus subtilis**

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Nonribosomal peptides are processed on multifunctional enzymes called nonribosomal peptide synthetases (NRPSs), whose modular multidomain arrangement allowed the rational design of new peptide products. However, the lack of natural competence and efficient transformation methods for most of nonribosomal peptide producer strains prevented the *in vivo* manipulation of these biosynthetic gene clusters. In this study, we present methods for the construction of a genetically engineered *Bacillus subtilis* surrogate host for the integration and heterologous expression of foreign NRPS genes. In the *B. subtilis* surrogate host, we deleted the resident 26-kilobase *srfA* gene cluster encoding the surfactin synthetases and subsequently used the same chromosomal location for integration of the entire 49-kilobase bacitracin biosynthetic gene cluster from *Bacillus licheniformis* by a stepwise homologous recombination method. Synthesis of the branched cyclic peptide antibiotic bacitracin in the engineered *B. subtilis* strain was achieved at high level, indicating a functional production and proper posttranslational modification of the bacitracin synthetases BacABC, as well as the expression of the associated bacitracin *self-resistance* genes. This engineered and genetically amenable *B. subtilis* strain will facilitate the rational design of new bacitracin derivatives.

Nonribosomal peptides represent a large family of bioactive secondary metabolites produced by bacteria and fungi. Many of these peptides are pharmacologically important drugs like the immunosuppressive cyclosporin A or the antibiotics penicillin, vancomycin, and bacitracin. Other nonribosomally synthesized peptides like siderophores are associated with pathogenicity of microorganisms. The extraordinary variety of biological properties is a result of the enormous structural diversity in this group of natural products.

A vast set of substrates like amino acids and hydroxyl as well as carboxylic compounds are known to be incorporated into the peptide chain. In addition, the assembled residues can be further modified by epimerization, *N*-methylation, acylation, glycosylation, or heterocyclization. The final products display linear, cyclic, and/or branched peptide backbones (1, 2).

Nonribosomal peptide assembly is catalyzed by large multifunctional nonribosomal peptide synthetases (NRPSs).[1] Sequencing of several genes encoding NRPSs combined with recent biochemical and structural studies revealed a universal modular scaffold for these enzymes. Each module represents a functional unit including a full complement of active sites for recognition, activation, and incorporation of one constituent into the product. According to the multiple thiotemplate mechanism (3), in the first step, the adenylation (A)-domain recognizes and activates the cognate substrate by ATP hydrolysis to the corresponding adenylate. Subsequently, the activated substrate is covalently linked onto the 4′-phosphopantetheinyl (4′-PPan) cofactor, which is attached to an invariant serine residue of the peptidyl carrier protein domain (PCP), that is located downstream of the A-domain. Posttranslational modification of the PCP-domain is catalyzed by a 4′-PPan-transferase (PPTase) (4). Normally, genes encoding PPTases are associated with most NRPS biosynthetic gene clusters. During the elongation reaction the PCP-tethered precursors are coupled to the nascent peptide chain by the condensation (C)-domain, which is located between each consecutive pair of activating units. In addition to the A-, PCP-, and C-domains, modifying domains are found in modules that incorporate modified residues. Final release of the full-length peptide chain by cyclization or hydrolysis is catalyzed by a thioesterase-like (Te)-domain residing at the COOH terminus of the last NRPS. As a consequence of these assembly line mechanisms, the primary sequence and the extent of modification of the final NRPSs product are controlled by a linear sequence of catalytic domains and modules (2, 5, 6).

Based on the modular arrangement of NRPSs, engineered manipulation of the nonribosomal protein templates enabled the rational design of new peptide products. Some of the strategies that have been devised were shown to yield functional hybrid templates *in vitro* (7, 8). The directed replacement of minimal modules (A-PCP-domains) (9, 10) as well as whole modules (11) in the surfactin biosynthesis operon (*srfA*) of *Bacillus subtilis in vivo* led to a predicted alteration of the amino acid sequence in the peptide product. Furthermore, truncated lipopeptide derivatives could be produced *in vivo* by moving the carboxyl-terminal intrinsic Te-domain (12) downstream of the internal PCP-domains of the *srfA* biosynthesis operon.

The cyclic branched peptide antibiotic bacitracin is produced by *Bacillus licheniformis* ATCC 10716, a strain that lacks

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF007865.

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[1] The abbreviations used are: NRPS, nonribosomal peptide synthetases(s); A-domain, adenylation domain; C-domain, condensation domain; PCP-domain, peptidyl carrier protein domain; Te-domain, thioesterase-like domain; ATCC, American Type Culture Collection; IU, international unit(s); 4′-PPan, 4′-phosphopantetheinyl; PPTase, 4′-phosphopantetheinyltransferase; kb, kilobase pair(s); bp, base pair(s); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; MS, mass spectroscopy; HPLC, high performance liquid chromatography.

TABLE I
Bacterial strains and plasmids

Strain	Relevant genotype/description	
B. subtilis		
KE10	Φ (*yckH-comS-erm-ycxA*)	
KE30	Φ (*yckH-comS-erm-ycxA)(amyE′-cat-p$_{spac}$-comS-lacI-′amyE*)	This work
KE300	Φ (*yckH-comS-bacT-kan-′bacB2′-ycxA)(amyE′-cat-p$_{spac}$-comS-lacI-′amyE*)	This work
KE310	Φ (*yckH-comS-bacT-bacA′-erm-′bacB′-ycxA)(amyE′-cat-p$_{spac}$-comS-lacI-′amyE*)	This work
KE320	Φ (*yckH-comS-bacT-bacA-bacB′-kan-′bacB′-ycxA)(amyE′-cat-p$_{spac}$-comS-lacI-′amyE*)	This work
KE340	Φ (*yckH-comS-bacT-bacA-C-bacR′-erm-′bcrABC-ycxA)(amyE′-cat-p$_{spac}$-comS-lacI-′amyE*)	This work
KE350	Φ (*yckH-comS-bacT-bacA-C-bacR′-kan-′bacS-bcrABC-ycxA)(amyE′-cat-p$_{spac}$-comS-lacI-′amyE*)	This work
KE360	Φ (*yckH-comS-bacT-bacA-C-bacRS-bcrABC-ycxA)(amyE′-cat-p$_{spac}$-comS-lacI-′amyE*)	This work
TS30	Φ (*srfA-C′-cat-′srfA-C*)	(9)
B. licheniformis		
ATCC 10716	Bacitracin producer	ATCC
AK1	Φ (*bacB′-kan-′bacB*)	(21)
AK20	Φ (*bacR′-kan-′bacS*)	(22)
M. luteus		
ATCC 10240	Wild type	ATCC
E. coli		
XL1Blue	*EndA1, gyrA96, hsdR17, lac, recA1, relA1, supE44, thi-1, F′(proAB, lacI^q, lacZΔM15,* Tn10(tet^r))	(46)
Plasmids		
pCm::Tc	Plasmid conferring Tc resistance	(25)
pDG646	Plasmid carrying the *erm*	(41)
pDG783	Plasmid carrying the *kan*	(41)
pDR66	*amyE* integration vector	(20)
pΔTE	Φ (*srfA-C′-cat-ycxA*)	(42)
pKE19	*SrfA* deletion plasmid Φ (*yckGH-comS-erm-ycxA*)	This work
pKE27	*comS* integration plasmid Φ (*amyE′-cat-p$_{spac}$-comS-lacI-′amyE*)	This work
pKE78	Φ (*yckGH-comS-bacT-bacA′-erm-′bacB′-ycxA*)	This work
pKE110	Φ (*bacB′-erm-bcrABC-ycxA*)	This work

natural competence. Genetic manipulations of the bacitracin biosynthesis operon have therefore so far been difficult to realize. However, the structural properties of the branched cyclic, thiazoline ring-containing peptide make bacitracin a desirable task to employ the genetic engineering strategies devised for NRPSs (13). Therefore, we constructed a heterologous expression system for the entire 49 kb comprising bacitracin biosynthesis operon (*bac*) in the genetically accessible surrogate host *B. subtilis*. The 26 kb comprising surfactin biosynthesis operon was deleted from the chromosome of *B. subtilis*, and the *bac* operon was integrated stepwise at the same chromosomal location by homologous recombination. Heterologous expression of the bacitracin biosynthesis gene cluster in the surrogate host *B. subtilis* revealed the production of the three NRPSs BacABC in a functional and posttranslational modified holo-form and expression of the bacitracin *self-resistance* genes. Furthermore, the heterologous *B. subtilis* host revealed an elevated bacitracin production compared with the wild type producer *B. licheniformis* ATCC 10716.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Media—Bacterial strains used in this investigation are listed in Table I. Cells were grown in 2× YT medium (38), in Difco Sporulation medium (39), or in modified SpII medium (40). For *Bacillus*, cultures supplemented with 25 μg/ml erythromycin, 1 μg/ml lincomycin, 5 μg/ml chloramphenicol, 10 μg/ml tetracycline, and 10 μg/ml kanamycin were used. In *Escherichia coli* final concentrations of 25 μg/ml kanamycin and 100 μg/ml ampicillin were used.

Plasmid Construction—DNA was amplified from chromosomal DNA of *B. licheniformis* ATCC 10716 if not indicated otherwise. PCR amplification was performed using the Expand long template PCR system (Roche, Mannheim, Germany) following the manufacture's protocol. Restriction sites for subsequent cloning were introduced with oligonucleotides, purchased from MWG-Biotech (Ebersberg, Germany) (listed in Table II). PCR products were purified with QIAquick-spin PCR purification kit (Qiagen, Hilden, Germany). Standard procedures were applied for all DNA manipulations (38).

A 2526-bp DNA fragment was amplified from chromosomal DNA of *B. subtilis* ATCC 21332 comprising the immediate upstream region of the *srfA* operon containing *yckG* and *yckH* using the oligonucleotides 5′*srfA*-OP(*Cla*I) and 3′*srfA*-OP(*Pst*I). The fragment was terminally modified using the endonucleases *Cla*I and *Pst*I and subsequently ligated into pΔTE, a derivative of pBluescript SK(II) (Stratagene, Amsterdam, Netherlands), cut in the same manner, to give pKE17. From chromosomal DNA of *B. subtilis* ATCC 21332, a 191-bp DNA fragment comprising the competence regulator gene *comS* was amplified using the oligonucleotides 5′*comS*(*Pst*I) and 3′*comS*(*Sph*I), terminally modified using the endonucleases *Pst*I and *Sph*I, and ligated into pKE17 previously cut in the same manner to give pKE18. An *erm* resistance cassette, obtained from pDG646 (41) by digest with *Sph*I, *Eco*RI, was ligated into pKE18, cut likewise, thereby replacing the *cat* resistance cassette to give pKE19.

A 195-bp DNA fragment comprising the gene *comS* was amplified from chromosomal DNA of *B. subtilis* ATCC 21332 using the oligonucleotides 5′*comS*(*Hin*dIII) and 3′*comS*(*Xba*I) and terminally modified using the endonucleases *Hin*dIII and *Xba*I. pDR66 (20) was cut in the same manner. Both fragments were ligated to give pKE27.

Using the oligonucleotides 5′*bacB*(*Eco*RI) and 3′*bacB*(*Bam*HI), a 1187-bp internal DNA fragment of *bacB* was amplified, terminally modified using the endonucleases *Eco*RI and *Bam*HI, and ligated into pKE18 previously cut in the same manner to give pKE61. A 1182-bp DNA fragment was amplified comprising the 5′ region of *bacT* using the oligonucleotides 5′*bacT*(*Sph*I) and 3′*bacT*(*Eco*RI). The fragment was terminally modified using the endonucleases *Sph*I and *Eco*RI and subsequently ligated into pKE61, cut in the same manner, to give pKE62. A *kan* resistance cassette, obtained from pDG783 (41) by digest with *Eco*RI, was ligated into pKE62, cut likewise to give pKE64.

An *erm* resistance cassette, obtained from pDG646 (41) by digest with *Sph*I and *Eco*RI, was ligated into pKE64, cut likewise, thereby replacing the *kan* resistance cassette to give pKE65. A 3101-bp DNA fragment comprising the 5′ region of *bacT* using the oligonucleotides 5′*homobacT*(*Pst*I) and 3′*homobacT*(*Sph*I) was amplified and terminally modified using the endonucleases *Pst*I and *Sph*I. pΔTE (42) was cut in the same manner. Both fragments were ligated to give pKE66. A 3791-bp DNA fragment containing the resistance cassette *erm*, *bacB*, and *ycxA* obtained from pKE65 by digest with *Sph*I and *Spe*I was ligated into pKE66, cut likewise to give pKE78.

With the oligonucleotides 5′*bacB1*(*Pst*I) and 3′*bacB1*(*Sph*I), a 2006-bp internal DNA fragment of *bacB* was amplified, terminally modified using the endonucleases *Pst*I and *Sph*I, and ligated into pΔTE (42) previously cut in the same manner to give pKE107. A 2261-bp DNA fragment was amplified comprising the ABC transporter genes *bcrABC* using the oligonucleotides 5′*bcr*(*Eco*RI) and 3′*bcr*(*Bam*HI). The frag-

TABLE II
Primers used in this study
Cloning restriction sites are in boldface type; modified sequences are in italics.

Oligonucleotides	Sequence
5′*srfA-OP*(*Cla*I)	5′-ATA ***ATC GAT*** AAA GAA TTT TAG TTC CTA GCT TC-3′
3′*srfA-OP*(*Pst*I)	5′-ATA ***CTG CAG*** CCT CCC CTA ATC TTT ATA A-3′
5′*comS*(*Pst*I)	5′-A*TT* ***CTG CAG*** CGT *ATG* AAC CGA TCA GG-3′
3′*comS*(*Sph*I)	5′-TA*T* ***GCA TGC*** CTG AAT TGC GTT TTC AAG-3′
5′*comS*(*Hin*dIII)	5′-*TAA* ***AAG CTT*** AGG AGG AGC AGA CGT ATG AAC-3′
3′*comS*(*Xba*I)	5′-*TTA* ***TCT AGA*** CGT TTT CAA GCC GGT CTT TAT-3′
5′*bacB*(*Eco*RI)	5′-TGT C***GA ATT C***CT CGG GAG AAT CGA T-3′
3′*bacB*(*Bam*HI)	5′-TAT ***GGA TCC*** ATC TTT CCT TTG GAT ATC TC-3′
5′*bacT*(*Sph*I)	5′-TAT ***GCA TGC*** TCC TGG CGC TGA TTG-3′
3′*bacT*(*Eco*RI)	5′-TAT ***GAA TTC*** AAG CGT CCG AAG AAG GAA-3′
5′*homobacT*(*Pst*I)	5′-TAT ***CTG CAG*** TCC TGG CGC TGA TTG-3′
3′*homobacT*(*Sph*I)	5′-TAT ***GCA TGC*** CAG CTT TTC TTC CGT TTC-3′
5′*bacB1*(*Pst*I)	5′-TTA ***CTG CAG*** GCA TGG AGA CAA CCT GAA-3′
3′*bacB1*(*Sph*I)	5′-TTA ***GCA TGC*** GAG CGG AAG AAA GCG-3′
5′*bcr*(*Eco*RI)	5′-TAT ***GAA TTC*** GCG ATC GAT GAA GTA TTG GAG-3′
3′*bcr*(*Bam*HI)	5′-TAT ***GGA TCC*** GCA CTC GAC AGA CCG T-3′

ment was terminally modified using the endonucleases *Bam*HI and *Eco*RI and subsequently ligated into pKE18, cut in the same manner, to give pKE108. An *erm* resistance cassette, obtained from pDG646 (41) by digest with *Sph*I and *Eco*RI, was ligated into pKE108, cut likewise, thereby replacing the *cat* resistance cassette to give pKE109.

A 4852-bp DNA fragment containing the resistance cassette *erm*, *bcrABC*, and *ycxA* obtained from pKE109 by digest with *Sph*I and *Spe*I was ligated into pKE107, cut likewise to give pKE110.

B. subtilis Strain Construction—The corresponding strains of *B. subtilis* were transformed by treatment with 10 ng of linearized plasmid or chromosomal DNA at an optical density of A_{600} 0.55 as described by Klein *et al*. (43) and plated on Difco Sporulation solid medium supplemented with an appropriate amount of antibiotic for selection. Loss or gain of antibiotic resistance were verified by replica plating.

Transformation of *B. subtilis* TS30 (9) with the *srfA* deletion plasmid pKE19 resulted in *B. subtilis* strain KE10 with the phenotype MLS^S and Cm^R (see Fig. 2). Transformation of the *B. subtilis srfA* deletion strain KE10 with the integration plasmid pKE27 resulted in *B. subtilis* strain KE30 with the phenotype Cm^R and MLS^R, harboring a second copy of *comS* within the *amyE* site.

B. subtilis KE30 was transformed with the plasmid pKE64 to give strain *B. subtilis* KE300 with the phenotype Cm^R, Km^R, and MLS^S. Transformation of *B. subtilis* KE300 with the plasmid pKE78 resulted in *B. subtilis* KE310 with the phenotype Cm^R, MLS^R, and Km^S (see Fig. 3). Thereby, the 5′ homologous region of *bacA* was extended from 1 kb (KE300) to 3.1 kb (KE310). Chromosomal DNA of *B. licheniformis* AK1 (21) was transformed into *B. subtilis* KE310, resulting in the *bacA* expression strain *B. subtilis* KE320 with the phenotype Cm^R, Km^R, and MLS^S (see Fig. 3).

Transformation of *B. subtilis* KE320 with the plasmid pKE110 resulted in *B. subtilis* KE340 with the phenotype Cm^R, MLS^R, and Km^S (see Fig. 5). Chromosomal DNA of *B. licheniformis* AK20 (22) was transformed in *B. subtilis* KE340, resulting in *B. subtilis* strain KE350 with the phenotype Cm^R, Km^R, and MLS^S, harboring the entire *bacRS* disrupted *bac* operon (see Fig. 5).

In the congression experiment, *B. subtilis* KE350 was transformed with chromosomal DNA of *B. licheniformis* ATCC 10716 together with the self-replicable helper plasmid pCm::Tc, resulting in *B. subtilis* strain KE355 with the phenotype Cm^R, Tc^R, and Km^S (see Fig. 6). After loss of the plasmid pCm::Tc (25), *B. subtilis* KE360 with the phenotype Cm^R, Tc^S, and Km^S could be obtained, harboring *bacTABCRS* and *bcrABC* within the former *srfA* locus (see Fig. 6).

Partial Purification of BacA from B. subtilis KE320—A prewarmed 400-ml volume of 2× YT medium was inoculated 1/100 with an overnight culture of the corresponding *Bacillus* strain and allowed to grow at 37 °C under aerobic conditions. Cells were harvested 2 h after entry into the stationary growth phase. Cells were resuspended in 5 ml of sucrose buffer A (20% sucrose, 50 mM Tris/HCl, 1 mM EDTA, 5 mM dithioerythritol, lysozyme 1 mg/ml pH 7.8) and subsequently incubated at 37 °C for 45 min. Protoplasts were broken by three passages through a French pressure cell (Amicon). The supernatant was separated by centrifugation and subjected to a 35–55% $(NH_4)_2SO_4$ precipitation. All following procedures were carried out at 4 °C. The precipitate was pelleted by centrifugation, resuspended in 2 ml of sucrose buffer B (10% sucrose, 50 mM Tris/HCl, 1 mM EDTA, 5 mM dithioerythritol, pH 8.0). The solution was applied to a 16/75 Sephacryl™ S-500 column (Amersham Pharmacia Biotech, Freiburg, Germany) previously equilibrated with sucrose buffer B. The flow rate was 1 ml/min. Elution was performed isocratically. Fractions containing the recombinant protein were identified by SDS-PAGE and subsequently pooled.

ATP-PP_i Exchange Assay—In order to test the activity to form aminoacyl adenylates, the amino acid-dependent ATP-PP_i exchange reaction was performed as described previously with minor modifications (44). The assay mixture contains 140 μl (~400 nM) of protein solution, 1 mM amino acid, 1 mM dATP, 50 μM PP_i and 20 mM $MgCl_2$ in sucrose buffer B. Exchange was initiated by the addition of 0.15 μCi of sodium [^{32}P]pyrophosphate in a total volume of 0.2 ml.

Assay for Detection of Covalent Amino Acid Incorporation—Thioester formation to detect covalently tethered amino acids and the degree of holo enzyme formation was carried out as described previously with minor modifications (44). The reaction mixture contains 180 μl (~500 nM) of protein solution, 1 mM ATP, 20 mM $MgCl_2$, and radiolabeled amino acid (purchased from Hartmann, Braunschweig, Germany; 3.4 μM L-[^{14}C]leucine (292 Ci/mol), 7.6 μM L-[^{14}C]isoleucine (260 Ci/mol), or 4.0 μM L-[^{14}C]proline (246 Ci/mol), respectively) in sucrose buffer B.

Surfactin Preparation and Detection—The lipopeptide surfactin (see Fig. 1) was extracted as described previously (10). The hemolytic activity of surfactin was analyzed using blood-agar plates (39).

The extracts were analyzed by HPLC/MS (Hewlett Packard 1100 Series, CC250/3 Nucleosil 120–3C^8 column, Macherey & Nagel, Düren, Germany) and monitored at 214 nm as well as in negative-ion mode over the m/z range from 900 to 1200. The following gradient profile was used at a flow rate of 0.3 ml min^{-1}: applying sample at 70% buffer B, performing a linear gradient to 100% buffer B in 30 min (buffer A, 0.05% formic acid in H_2O; buffer B, 0.045% formic acid in methanol).

Preparation and Detection of Bacitracin—Bacitracin was prepared as described previously using 2× YT medium instead of M20 medium (22). The activity of the extracts were analyzed by the use of freshly prepared *Micrococcus luteus* plates (45). After incubation overnight at 37 °C, *M. luteus* growth inhibition zones were measured and compared with each other.

The extracts were further analyzed by HPLC/MS (Hewlett Packard 1100 Series, Sephasil TM C^{18} column (5 μm; 250 × 4 mm; Amersham Pharmacia Biotech)) monitored at 214 and 253 nm. Scans were taken in positive-ion mode over the m/z range from 600 to 1600. The following gradient profile was used at a flow rate of 0.2 ml min^{-1}: applying sample at 25% buffer B, performing a linear gradient to 50% buffer B in 60 min, following a linear gradient to 100% buffer B in 5 min (buffer A, 0.01% trifluoric acid in H_2O; buffer B, acetonitrile).

RESULTS

Construction of the B. subtilis ATCC 21332 srfA Deletion Strain KE10—Here, we report the construction of a heterologous expression strain derived from the genetically accessible surfactin producer *B. subtilis* ATCC 21332 (14). Due to the instability of plasmids containing large insertions of foreign DNA in *B. subtilis* (15, 16), expression of giant recombinant gene clusters like those coding for NRPSs can only be achieved by a stable chromosomal integration.

To avoid the expansion of the *B. subtilis* chromosome by

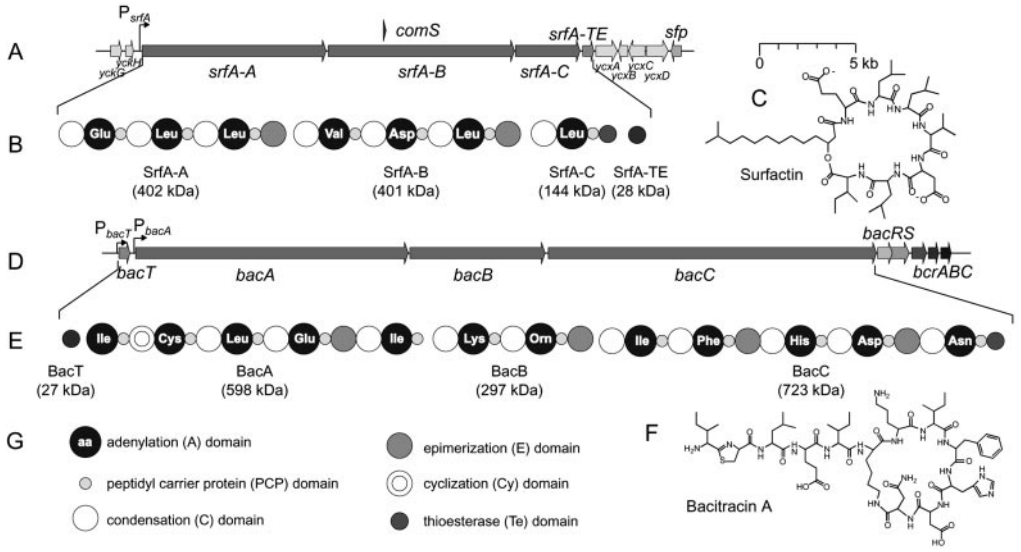

FIG. 1. **The surfactin and bacitracin biosynthesis gene clusters.** *A*, the chromosomal region of the genes of the surfactin biosynthesis operon (*srfA*) from *B. subtilis* ATCC 21332. Four genes *srfA-ABCTE* encode the surfactin peptide synthetases SrfA-A, SrfA-B, and SrfA-C as well as the external thioesterase SrfA-TE (*B*) that assemble the lipoheptapeptide surfactin (*C*). *D*, the chromosomal region of the bacitracin biosynthesis gene cluster (*bac*) from *B. licheniformis* ATCC 10716 containing the genes *bacTABCRS* and *bcrABC*. The three bacitracin peptide synthetases BacABC (*E*) assemble the peptide mixture with the main component bacitracin A (*F*). In *G*, the code of the patterns used for the different domains is shown.

integration of NRPSs biosynthesis gene clusters of enormous size, we have first deleted the resident 26 kb spanning surfactin biosynthesis operon (*srfA*) (see Fig. 1) by a single homologous recombination event. The deletion of the *srfA* operon also led to the deletion of a small competence regulator gene designated *comS* residing in a different reading frame within the first module of *srfA-B* (see Fig. 1), which is indispensable for the development of native competence in *B. subtilis* (17, 18).

For the construction of the *srfA* deletion plasmid pKE19, the upstream homologous region of *srfA* (2.5 kb) containing *srfA* operator, promoter, and RBS was cloned (see Fig. 2). To obtain a *B. subtilis srfA* deletion strain that still maintains its genetic competence, the transcriptional control of *comS* (138 bp) was restored by fusion to the RBS of the *srfA* promotor. Its start codon was altered from TTG to ATG to facilitate an efficient initiation of translation. The downstream homologous region of *srfA* (939 bp) (see Fig. 2) was determined in a way that the transcriptional regulation of the succeeding open reading frame *ycxA* (1.2 kb) was restored.

For transformation we used the *B. subtilis* derivative TS30 (9), containing a *cat* resistance marker in the *srfA-C* site, that permits screening on double crossover integration (see Fig. 2). After transformation of *B. subtilis* TS30 with the *srfA* deletion plasmid pKE19, three transformants with the phenotype MLS^R and Cm^S were selected. The correct integration was confirmed by Southern blotting analysis, which revealed the correct substitution by double crossover (data not shown). The resulting strain carrying the deletion of the entire *srfA* operon (26.1 kb) including the surfactin synthetases A-C and *srfA-TE* was designated KE10 (see Fig. 2). The incapacity of *B. subtilis* KE10 to produce surfactin was demonstrated by non-hemolytic activity of cell broth on blood agar plates as well as by HPLC/MS analysis (data not shown).

Integration of comS into the amyE site—It has been reported that competence gene transcription and transformation efficiency in *B. subtilis* can be increased using multicopy expression of the competence regulator *comS* (19). Following this approach, we integrated a second copy of *comS* into the α-amylase encoding site of the chromosome to enhance transformation efficiency.

ComS (138 bp) was cloned under the control of the isopropyl-

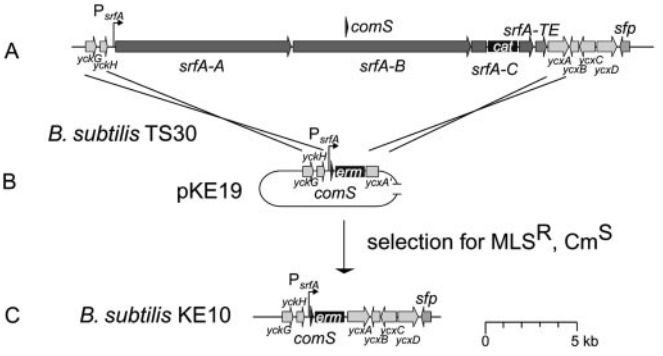

FIG. 2. ***SrfA* deletion in *B. subtilis* TS30: schematic diagram showing the deletion of the *srfA* locus in *B. subtilis* TS30 (9).** *A*, the chromosomal *srfA* locus of *B. subtilis* TS30. Transformation of *B. subtilis* TS30 with the plasmid pKE19 (*B*) and homologous recombination resulted in the *srfA* deletion strain *B. subtilis* KE10 (*C*). Note the repositioning of *comS* under the *srfA* promotor upon deletion of the entire 26-kb operon.

1-thio-β-D-galactopyranoside-inducible *spac* promoter in the *Bacillus amyE* integration vector pDR66 (20) resulting in pKE27 (see "Experimental Procedures"). Transformation of the *B. subtilis srfA* deletion strain KE10 with the integration plasmid pKE27 resulted in five transformants with the phenotype Cm^R and MLS^R. The correct integration via double crossover was confirmed using Southern hybridization (data not shown). The resulting *srfA* deletion strain harboring a second copy of *comS* within the *amyE* chromosomal site was designated KE30. Investigations of competence efficiency demonstrated a satisfactory transformation rate of the constructed *B. subtilis* strain KE30 (data not shown).

Integration of bacT and bacA in B. subtilis KE30—The nonribosomal biosynthesis of bacitracin was accomplished by three large multienzymes comprising 12 modules: the peptide synthetases BacA (598 kDa), BacB (297 kDa), and BacC (723 kDa) (see Fig. 1). The encoding genes *bacA* (15.7 kb), *bacB* (7.8 kb), and *bacC* (19.0 kb) are organized in an operon (21). Upstream of *bacABC*, *bacT* (705 bp) is found encoding an external thioesterase (see Fig. 1). Recent investigations concerning the *bacABC* downstream region revealed genes encoding a two

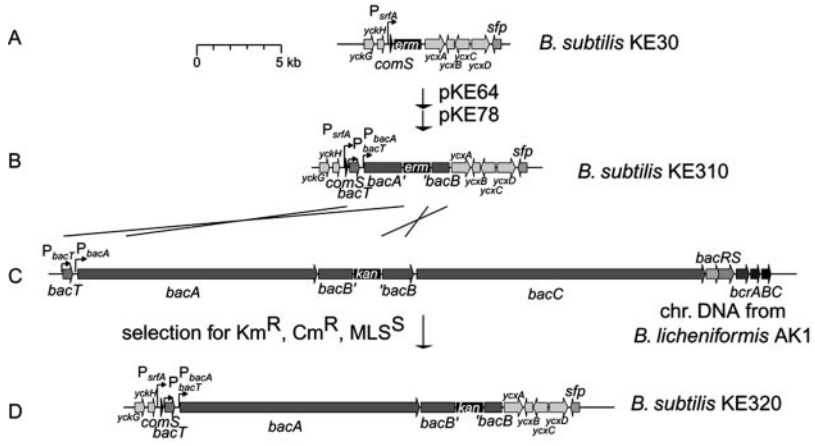

FIG. 3. **Integration of *bacT*, *bacA*, and *bacB1* in *B. subtilis* KE30.** Schematic diagram showing the construction of *B. subtilis* KE320. *A*, the former *srfA* site in the chromosome of *B. subtilis* KE30. Transformation of *B. subtilis* KE30 with pKE64 and pKE78 and homologous recombination resulted in *B. subtilis* KE310 (*B*). Transformation of *B. subtilis* KE310 with chromosomal DNA of *B. licheniformis* AK1 (*C*) and homologous recombination resulted in the chromosomal integration of *bacT*, *bacA*, and *bacB1* (24 kb) in *B. subtilis* KE320 (*D*).

component system (*bacR* (717 bp) and *bacS* (1047 bp)) and an ABC transporter (*bcrA* (921 bp), *bcrB* (627 bp), and *bcrC* (612 bp)) (see Fig. 1) that were shown to be associated with *self-resistance* to bacitracin (22). Therefore, genes for bacitracin biosynthesis as well as bacitracin resistance are located within a unique chromosomal region of 49 kb of *B. licheniformis* ATCC 10716.

The integration of the entire bacitracin biosynthesis operon under the transcriptional control of the native σ^A-dependent *bac* promotor was achieved in two steps. For integration of the first 24-kb 5′-region comprising *bacT*, *bacA*, and a disrupted *bacB* fragment into the former *srfA* locus of the chromosome, a derivative of *B. subtilis* KE30 was used (see Fig. 3). The strain *B. subtilis* KE310 (see "Experimental Procedures"), harboring a short 5′ region (3.1 kb) containing *bacT* and *bacA1* and a short 3′ region (1.1 kb) of *bacB*, was transformed with chromosomal DNA of *B. licheniformis* AK1 (21), carrying a *kan* resistance marker interrupting *bacB* downstream of the first module, which allows screening on integration by monitoring resistance toward kanamycin. Three transformants with the phenotype Cm^R, Km^R, and MLS^S were identified (see Fig. 3). Correct chromosomal integration of the 24-kb DNA fragment was confirmed by Southern hybridization (data not shown). The obtained *B. subtilis* strain, which was shown to produce the 598-kDa BacA protein in significant amounts during the transition state growth phase, was designated KE320 (see Fig. 4*A*). In this recombinant strain KE320, an improvement of BacA production, whose gene is regulated by the σ^A-dependent *bac* promotor in *B. subtilis*, was observed.

Partial Purification and in Vitro Analysis of BacA from B. subtilis KE320—BacA (598 kDa) was partially purified using $(NH_4)_2SO_4$ precipitation and size exclusion chromatography (see Fig. 4*B*). The activity of the protein could be demonstrated by activation of substrate amino acids and thioester formation assay.

The predicted specificity of BacA toward L-isoleucine, L-cysteine, and L-leucine was confirmed; however, no specificity toward L-glutamate was observed (see Fig. 4*C*). Investigations concerning the specificity of the wild type protein BacA purified from *B. licheniformis* ATCC 10716 also revealed no activation of L-glutamate (23).

Thioester formation of the substrate amino acids L-isoleucine and L-leucine was also demonstrated (see Fig. 4*D*), indicating a proper posttranslational modification of BacA's PCP-domains with the cofactor 4′-PPan. Likely, the resident *B. subtilis* PPTase Sfp (24) is responsible for holo-BacA formation.

Integration of bacB, bacC, and bcrABC in B. subtilis KE320—The integration of the entire bacitracin biosynthesis operon was completed according to Fig. 5. For the second integration step, the 30-kb DNA fragment comprising the 3′ region

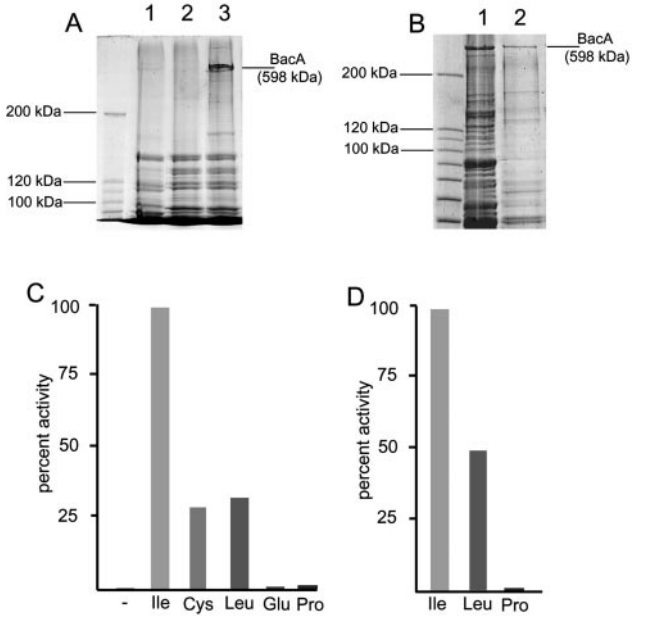

FIG. 4. **Partial purification and characterization of BacA from *B. subtilis* KE320.** *A*, Coomassie Blue-stained SDS-PAGE showing the production of BacA (598 kDa) in *B. subtilis* KE320. *Lane 1*, total cellular proteins of *B. licheniformis* ATCC 10716; *lane 2*, total cellular proteins of the *srfA* deletion strain *B. subtilis* KE30; *lane 3*, total cellular proteins of heterologous BacA producer *B. subtilis* KE320. *B*, Coomassie Blue-stained SDS-PAGE showing partial purification of heterologously produced protein BacA from *B. subtilis* KE320. *Lane 1*, proteins after subjection to a 35–55% $(NH_4)_2SO_4$ precipitation; *lane 2*, proteins purified on size exclusion chromatography (Sephacryl™ S-500). *C*, substrate specificity of heterologously produced BacA investigated by the ATP/PP_i-exchange reaction. BacA showed a specific activation of the substrate amino acids L-isoleucine, L-cysteine, and L-leucine. No activation was found for the cognate amino acid L-glutamate and the non-cognate amino acid L-proline. The highest activation rate for L-isoleucine was defined as 100%. *D*, thioester formation assay showed a significant tethering of radiolabeled substrate amino acids L-[^{14}C]isoleucine and L-[^{14}C]leucine into BacA. The non-cognate amino acid L-[^{14}C]proline was not incorporated. The highest incorporation rate for L-isoleucine was defined as 100%.

of *bacB*, *bacC*, as well as *bcrABC* coding for the ABC transporter were integrated using a derivative of *B. subtilis* KE320. *B. subtilis* KE340 (see "Experimental Procedures") harbors a 5′ region (20.6 kb) containing *bacTAB1* and the short 3′ region (2.2 kb) *bcrABC* in the former *srfA* locus (see Fig. 5). Transformation with chromosomal DNA of *B. licheniformis* AK20 (22), containing a *kan* resistance marker in the disrupted two-component system *bacRS*, which permits screening on double crossover integration, resulted in two transformants with the phenotype Cm^R, Km^R, and MLS^S (see Fig. 5). Correct chromo-

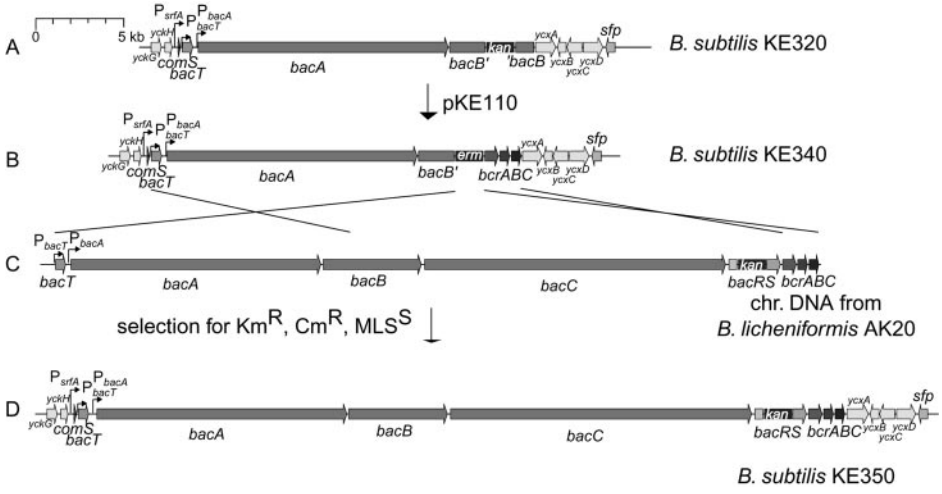

FIG. 5. **Integration of the entire *bac* gene cluster in *B. subtilis* KE320.** Schematic diagram shows the entire integration of the 49-kb bacitracin biosynthesis gene cluster in *B. subtilis* KE320. *A*, the corresponding chromosomal locus in the heterologous BacA producer strain *B. subtilis* KE320. Transformation of *B. subtilis* KE320 with pKE110 and homologous recombination resulted in *B. subtilis* KE340 (*B*). Transformation of *B. subtilis* KE340 with chromosomal DNA of *B. licheniformis* AK20 (*C*) and homologous recombination resulted in the chromosomal integration of the entire *bacRS* interrupted *bac* biosynthesis gene cluster. This strain was designated *B. subtilis* KE350 (*D*).

somal integration of the 30-kb DNA fragment was confirmed by Southern hybridization (data not shown). The obtained *B. subtilis* strain harboring *bacTABC* and *bcrABC* in the former *srfA* locus was designated KE350 (see Fig. 5). SDS-PAGE analysis confirms the production of the three nonribosomal peptide synthetases BacABC in significant amounts during the transition state growth phase (see Fig. 7). In comparison to the bacitracin producer strain *B. licheniformis* ATCC 10716, an improvement of BacABC production in *B. subtilis* was demonstrated. Production of the bacitracin ABC transporter BcrABC was demonstrated using Western blot analysis (data not shown).

Reconstitution of the Two-component System bacRS—Recently, the influence of the two-component system BacRS on the expression of *bcrABC*, conferring bacitracin *self-resistance* in the producer strain *B. licheniformis,* has been demonstrated (22). Therefore, the gene cluster of *bacRS* located downstream of the bacitracin biosynthesis operon was reconstructed in *B. subtilis* KE350 (see Fig. 6). This was achieved by a *marker exchange* replacement (13), yielding in *B. subtilis* KE360. Transformation of *B. subtilis* KE350 with chromosomal DNA of *B. licheniformis* ATCC 10716 and the self-replicable helper plasmid pCm::Tc (25) resulted in two transformants with the phenotype Cm^R, Tc^R, and Km^S (see Fig. 6). Upon loss of the plasmid pCm::Tc, transformants with the phenotype Cm^R, Tc^S, and Km^S were isolated (see Fig. 6). Correct integration of the intact *bacRS* was confirmed by Southern hybridization (data not shown). The obtained strain harboring the entire bacitracin biosynthesis gene cluster residing in the former *srfA* locus was designated *B. subtilis* KE360 (see Fig. 6). In this strain, the three nonribosomal peptide synthetases BacABC were detected by SDS-PAGE (see Fig. 7). The production of the bacitracin-sensing proteins BacRS and the ABC transporter BcrABC was confirmed by Western blot analysis (data not shown).

Heterologous Bacitracin Production in B. subtilis KE360—*B. subtilis* strain KE360 was shown to produce a mixture of cyclic bacitracins with the bioactive bacitracin A and its oxidation product bacitracin F as the main components (26) (see Fig. 8). Due to the indefinite composition of the purchased bacitracin standard (Sigma, Deisenhofen, Germany) as well as the instability of bacitracin A against oxidation, the quantification of the major product bacitracin A was carried out based on its antibiotic activity against *Micrococcus luteus* (see "Experimental Procedures"). For the constructed *B. subtilis* strain KE360, a bacitracin A production in 2× YT medium of 8400 IU/liter was observed (see Table III). Comparison with the producer strain *B. licheniformis* ATCC 10716 (5600 IU/liter) indicated an increase in bacitracin A production of ~50%.

The composition of the heterologously produced bacitracin mixture was analyzed using HPLC/MS (see Fig. 8). The main compounds bacitracin A (at 36.5 min, M + 1 = 1422, 9 *m/z*, M + 2 = 712.2 *m/z*) and its oxidation product bacitracin F (at 58,0 min, M + 1 = 1419, 9 *m/z*, M + 2 = 710.2 *m/z*) were detected in the extract of *B. subtilis* KE360 (see Table III and Fig. 8).

No bacitracin-related inhibition of cell-growth was observed for *B. subtilis* KE360, since heterologous co-expression with *bacRS* and *bcrABC* led to a 14-fold increase in bacitracin self-resistance compared with *B. subtilis* KE30 (see Table III). Only a bacitracin concentration of 280 IU/ml led to an inhibition of growth in the recombinant *B. subtilis* KE360 strain, whereas growth of the *srfA* deletion strain *B. subtilis* KE30 was already inhibited at 20 IU/ml.

DISCUSSION

In this study, we present the construction of a *B. subtilis* strain for the heterologous expression of the entire 49-kb bacitracin biosynthesis operon from *B. licheniformis* ATCC 10716. The heterologous production of the three peptide synthetases BacABC in a functional and posttranslational modified active holo-form, the conferral of bacitracin *self-resistance*, as well as the formation of the nonribosomal branched cyclic peptide antibiotic bacitracin are demonstrated.

Although members of the genus *Bacilli* have long been known to produce nonribosomal peptides, *B. subtilis* has never been used before as a host for recombinant NRPSs production. This fact likely refers to the described plasmid instability (15, 16). However, the ability to produce nonribosomal peptides and the well established genetic and fermentation methods for *B. subtilis* make it an attractive target for such studies. High level heterologous expression of the bacitracin biosynthesis operon (*bac*) in *B. subtilis* is maintained from a single chromosomal copy. Although the construction of chromosomal mutants is a time-consuming process, during fermentation no loss of genetic markers was observed, in contrast to the recently reported heterologous expression of the entire 6-deoxyerythronolide B from *Saccharopolyspora erythraea* based on a vector-host system in *E. coli* (27).

The integration of the entire bacitracin biosynthesis operon resulted in a 1.1% extension of the *B. subtilis* genome (28). However, due to the prior deletion of the resident 26-kb spanning surfactin biosynthesis operon, the chromosomal expansion was reduced to 0.5%. The deletion of the *srfA* operon containing the NRPSs genes *srfA-ABCTE,* simultaneously led to the deletion of a small competence regulator gene designated *comS* (138 bp), residing in a different reading frame within the first module of *srfA-B* (29). In the presence of ComS, the competence transcription factor ComK is activated to act as a

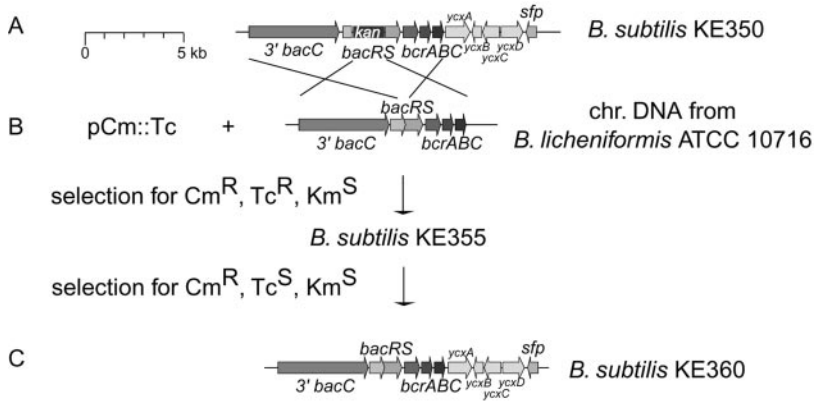

FIG. 6. **Reconstitution of the two-component system *bacRS* in *B. subtilis* KE350.** *A*, the chromosomal 3′*bac* locus of *B. subtilis* KE350. Simultaneous transformation of *B. subtilis* KE350 with chromosomal DNA of *B. licheniformis* ATCC 10716 (*B*) and the plasmid pCm::Tc yielded in the reconstitution of the two-component system *bacRS* (*B. subtilis* KE355). By screening on Tc sensitivity, the *B. subtilis* strain KE360 (*C*) was obtained.

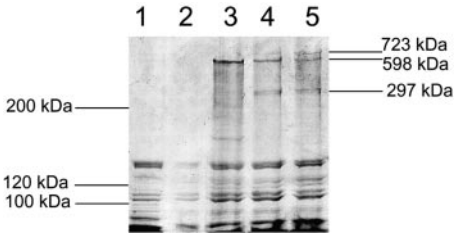

FIG. 7. **Production of the peptide synthetases BacABC.** Coomassie Blue-stained SDS-PAGE showing the production of the bacitracin peptide synthetases BacA (598 kDa), BacB (297 kDa), and BacC (723 kDa) in *B. subtilis* KE360. *Lane 1*, total cellular proteins of *B. licheniformis* ATCC 10716; *lane 2*, total cellular proteins of the *srfA* deletion strain *B. subtilis* KE30; *lane 3*, total cellular proteins of the BacA producer strain *B. subtilis* KE320; *lane 4*, total cellular proteins of *B. subtilis* KE350; *lane 5*, total cellular proteins of *B. subtilis* KE360.

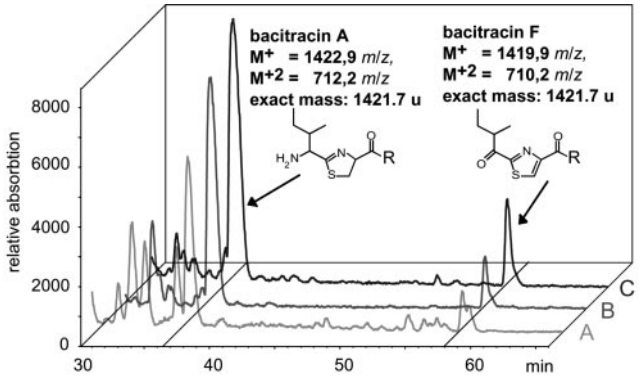

FIG. 8. **HPLC/MS analysis of the produced bacitracins.** HPLC/MS diagrams showing the bacitracin mixtures produced in *B. licheniformis* ATCC 10716 (*B*) and *B. subtilis* KE360 (*C*) compared with the purchased standard (*A*).

positive regulator for the transcription of the late competence genes encoding proteins involved in the synthesis and assembly of the DNA binding and uptake apparatus (17, 18, 30). The absence of ComS led to inactivation of ComK and loss of genetic accessibility of *B. subtilis*. Therefore, we have repositioned *comS* under the control of the *srfA* promotor (see Fig. 2) restoring the native competence cascade. In addition, a second chromosomal copy of *comS* was integrated under the control of the inducible *spac* promotor in the *amyE* site of the chromosome to improve the transformation efficiency (19). Following this strategy, the constructed *B. subtilis srfA* deletion strain KE30 maintained its genetic competence.

Only 17% of the 49 kb comprising bacitracin gene cluster have been amplified by PCR. For integration of the *bac* gene cluster, the main part of DNA has been inserted in the chromosome of *B. subtilis* by homologous recombination employing chromosomal DNA of *B. licheniformis*, minimizing the introduction of mutations.

TABLE III
Bacitracin production and resistance

Strains	Produced enzymes	Bacitracin quantification	Bacitracin qualification	Bacitracin resistance[a]
		IU/liter		*IU/ml*
B. licheniformis (ATCC 10716)	BacTABC BacRS BcrABC	5600	Bacitracin A (93%) Bacitracin F (7%)	280
B. subtilis (KE30)				20
B. subtilis (KE360)	BacTABC BacRS BcrABC	8400	Bacitracin A (87%) Bacitracin F (13%)	280

[a] 70 IU = 1 mg of bacitracin standard (Sigma, Deisenhofen, Germany).

Due to the enormous size of *multi*modular NRPSs, their heterologous expression was found to be impracticable in several cases due to instability and degradation (31). Moreover, proper folding of such multidomain enzymes and their proper posttranslational modification in foreign host is not predictable (27, 32, 33). None of these obstacles have been encountered in this study; the three bacitracin peptide synthetases BacA (598 kDa), BacB (297 kDa), and BacC (723 kDa) were heterologously produced at high levels and were posttranslationally modified to their active holo-forms (see Figs. 4 and 7).

In the native bacitracin producer strain *B. licheniformis* ATCC 10716, posttranslational modification of the peptide synthetases is likely to be catalyzed by the PPTase Bli (34), whose gene (*bli*) was found to be associated with the *lic* operon.[2] The PPTase gene associated with the surfactin synthetases in *B. subtilis*, *sfp*, is localized downstream of the former *srfA* operon gene locus in *B. subtilis* KE360 (24). A broad substrate tolerance of Sfp toward PCP-domains as well as the related acyl carrier proteins from polyketide synthases has been demonstrated (35). Recently, for heterologous posttranslational modification of the 6-deoxyerythronolide B synthases in *E. coli*, Sfp was successfully performed (27). Therefore, the resident *B. subtilis* PPTase Sfp is a good candidate for posttranslational modification of heterologously produced peptide synthetases.

Due to the sensitivity of the Gram-positive bacterium *B. subtilis* against the peptide antibiotic bacitracin, the expression of the *self-resistance* conferring genes encoding the two-component system BacRS (27.4 and 39.5 kDa) and the ABC transporter BcrABC (34.5, 23.3, and 23.1 kDa) (22, 36, 37) was an essential event. Thus, the constructed *B. subtilis* strain KE360 shows a bacitracin resistance comparable to the native producer *B. licheniformis*.

B. subtilis KE360 shows an ~50% elevated bacitracin A

[2] A. M. Neumüller and M. A. Marahiel, unpublished results.

production compared with the parental strain *B. licheniformis* (see Table III). This improvement in bacitracin productivity can be explained by the high level expression of the bacitracin synthetases, as well as the higher growth rate of the surrogate host *B. subtilis* KE360. As evaluated by HPLC/MS analysis, bacitracin A and F are the main compounds formed in *B. subtilis* KE360 as well as in *B. licheniformis* ATCC 10716 (see Table III). Therefore, the ratio of products seems to be determined rather by a strict substrate specificity than by a different substrate availability. On the other hand, the higher level of bacitracin F may be due to the cellular environment in *B. subtilis* that facilitates the oxidation of the peptide product.

So far, the genetic inaccessibility of the native bacitracin producer strain *B. licheniformis* ATCC 10716 has impeded the genetic engineering of the bacitracin biosynthesis gene cluster. With the construction of the heterologous *B. subtilis* expression system presented in this study, the engineered manipulation of the corresponding BacABC protein template can be envisioned. The BacABC protein template provides 12 modules with unique activities for 10 different substrate amino acids (see Fig. 1). Four epimerization domains of different specificity are found, as well as a thiazoline ring forming cyclization domain. The thioesterase domain of BacC seems to have an unique specificity to catalyze the formation of only one branched cyclic peptide backbone. The application of genetic strategies for recombination and alteration of individual domains or entire modules has been substantiated by the successful construction of simple model hybrid peptide synthetases *in vitro* (7, 8). Now, it is tempering to exploit these strategies to invent a vast set of bacitracin derivatives on demand.

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