Macrolactin is the Polyketide Biosynthesis Product of the pks2 Cluster of *Bacillus amyloliquefaciens* FZB42

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In the genome of *Bacillus amyloliquefaciens* FZB42, three operons *pks*1, *pks*2, and *pks*3 were identified which encode the biosynthesis of polyketides. *pks*1 and *pks*3 have been attributed to the production of bacillaene and difficidin/oxydifficidin, respectively, while the *pks*2 product remained hitherto unknown. Mass spectrometric analysis of the culture filtrates of the wild-type *B. amyloliquefaciens* FZB42 and mutants revealed *pks*2-specific metabolites. By combination of the mass spectrometric and UV/vis data with a database search, these compounds were attributed to four members of the macrolactin family, macrolactin A and D as well as 7-*O*-malonyl- and 7-*O*-succinyl-macrolactin. This conclusion was verified by the isolation and structure elucidation of macrolactin A using mass spectrometric and 2D-NMR studies. Macrolactin biosynthesis was investigated using feeding experiments with ^{13}C-acetate. ^{13}C-labelled macrolactin A revealed an alternating labelling of its carbon skeleton with ^{13}C, indicating that acetate/malonate was used as the sole precursor. The macrolactin structure is compatible with the domain organization of the *pks*2-operon. Similarly to *pks*1 and *pks*3, *pks*2 is a modular polyketide synthase system of type I which exhibits a *trans*-acyltransferase architecture using a discrete acyltransferase enzyme iteratively in the assembly of macrolactin. Finally, the potential for macrolactin production on a genetic and metabolic basis was found to be widely distributed among *Bacillus amyloliquefaciens* strains.

Throughout the past 50 years, the soil bacterium *Bacillus subtilis* has been used for genetic and biochemical studies and has become an important model system for Gram-positive bacteria. Several hundred wild-type *B. subtilis* strains have been collected with the potential to produce numerous antibiotics, predominantly peptides (e.g., the surfactins, the fengycins, and the iturins).[1] These lipopeptides are amphiphilic molecules that vary in their peptide or their fatty acid moiety.[2] Surfactins have strong hemolytic and surfactant properties[3] and show high antimycoplasma and antiviral activities.[4,5] Fengycins are potent antifungal agents,[6] and iturins, showing a great molecular diversity, differ in their hemolytic, antibacterial, or antifungal properties.[7–9] *B. subtilis* also produces some lantibiotics, two of type A (subtilin[1] and the ericins[10]) as well as three of type B (mersacidin,[1] sublancin,[11] and subtilosin[12,13]). The biosynthesis of all of these compounds has been widely explored, and the corresponding gene clusters have been identified.[1] There are two different pathways for the biosynthesis of peptides, the ribosomal synthesis of linear precursor peptides that are post-translationally modified and proteolytically processed (lantibiotics and lantibiotic-like peptides) and the nonribosomal mechanism at multimodular templates of peptide synthetases (e.g., surfactins, fengycins, iturins).[1,14]

Apart from peptides, polyketides are the other dominant family of secondary metabolites having antimicrobial, immunosuppressive, antitumor, or other physiologically relevant bioactivities. Although polyketides are widespread secondary metabolites from bacteria, only a few have been isolated and characterized from *Bacillus*. As a consequence, the corresponding biosynthetic gene clusters of polyketides in *Bacillus* spp. and their biosynthetic potential remained hitherto widely unexplored. Recently, we were able to assign the complete polyketide synthase (PKS) gene clusters to *Bacillus* antibiotics for the first time. This was done with the industrially relevant *B. amyloliquefaciens* FZB42 as a model organism.[15] FZB42 is a plant-root-colonizing environmental strain with the ability to stimulate plant growth and to suppress soil-borne plant pathogens in the rhizosphere.[16,17] Because of its beneficial action on plant development and biocontrol, it is of great interest in plant biotechnology. In the genome of this strain, three PKS operons were located at sites approximately 1.4 Mbp (*pks*2), 1.7 Mbp (*pks*1), and 2.3 Mbp (*pks*3) clockwise distant from the origin of replication in the *B. amyloliquefaciens* genome,[15] which is 3.916 kb in size.[18] All three gene clusters show a modular organization typical for type I PKS systems, implying that FZB42 has the biosynthetic machinery for the production of at least three different kinds of polyketides. With the help of cassette mutagenesis in combination with advanced mass spectrometric techniques, such as matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and high-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI-MS), two polyketides, bacillaene and difficidin/oxydifficidin (Figure 1), were identified, which are encoded by gene clusters *pks*1 (*bac*) and *pks*3 (*dif*).[15] These metabolites are also produced by the model organism *B. subtilis*.[2] Recently, the groups of Clardy et al. determined the structure of bacillaene from *B. subtilis* (Figure 1).[19]

In this paper, we report on the assignment of the polyketide family of macrolactins (Figure 1) as biosynthetic products of the *pks*2 cluster of *Bacillus amyloliquefaciens*. The macrolactins consist of 24-membered ring lactones with modifications such as the attachment of glucose β-pyranoside, or they occur as linear analogues.[20] Until now, at least 18 macrolactins have been identified, including seven with a molecular mass of 402 Da.[20,21] Originally the macrolactin family was isolated from an unclassified deep-sea bacterium including macrolactins A–F as well as the open-chain species macrolactinic and isomacrolactinic acid.[20] Macrolactin A was of particular interest, because of its interesting medical properties.[20] It shows selective antibacterial activities, the ability to inhibit B16-F10 murine melanoma cancer cells in *in vitro* assays as well as mammalian *Herpes simplex* viruses. In addition, it protects lymphoplast cells against HIV by inhibiting virus replica-

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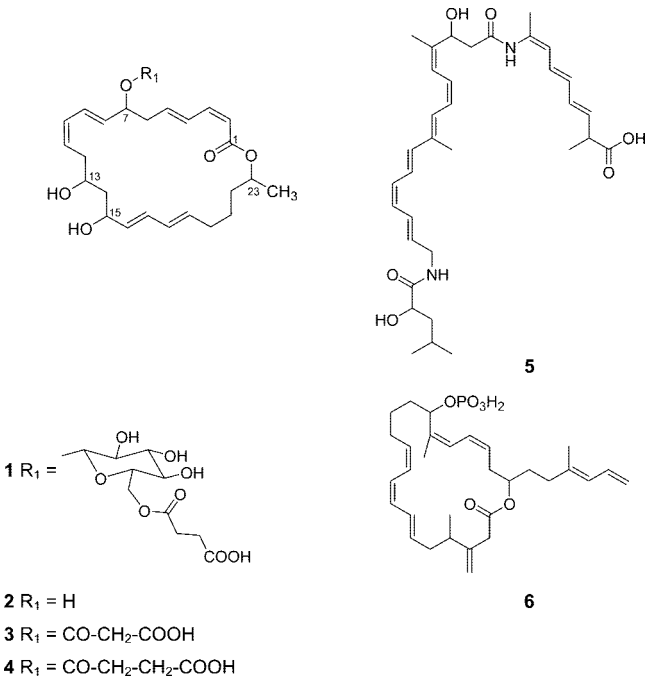

Figure 1. Structures of macrolactins (**1**–**4**), bacillaene (**5**), and difficidin (**6**), metabolites of *Bacillus* PKS biosynthetic systems.

tion.[20] Over the years, the macrolactin family has grown with the discovery of macrolactins G–M,[21] 7-*O*-malonyl macrolactin,[22] 7-*O*-succinyl macrolactin A,[23] and macrolactin N.[24] An interesting discovery was the antibacterial activities of 7-*O*-malonyl macrolactin against Gram-positive bacterial pathogens, in particular, its action against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci.[22] The determination of macrolactins as secondary metabolites of *B. amyloliquefaciens* allows the annotation of the *pks*2 gene cluster. Feeding experiments with ^{13}C-acetate show a full labelling of the macrolactin carbon skeleton and give insights into the nature of the starter and extender molecules. Finally, 30 *Bacillus* strains were tested for their production of the macrolactins, and the results of this screening led to the conclusion that these polyene antibiotics are widespread metabolites of *B. amyloliquefaciens*. The present contribution will aid in the understanding of polyketide biosynthesis in *Bacillus*.

Results and Discussion

To assign biological functions to the polyketide gene clusters in the genome of *B. amyloliquefaciens* FZB42, disruption of all three *pks* target genes was carried out by the insertion of an antibiotic resistance cassette via homologous recombination. Mutants were prepared bearing gene replacement mutations in the respective first KS domains of *pks*1 (CH6 [Δ*pks1KS1*::*cat*]), *pks*2 (CH7 [Δ*pks2KS1*::*cat*]), and *pks*3 (CH8 [Δ*pks3KS1*::*ermAM*]) and double-knockout mutants CH11 [Δ*pks1KS1*::*cat* Δ*pks3KS1*::*ermAM*]), CH12 [Δ*pks2KS1*::*cat* Δ*pks3KS1*::*ermAM*], and CH14 [Δ*pks1KS1*::*cat* Δ*pks2KS1*::*neo*].[15] The unknown products, specifically encoded by *pks*2, were detected mass spectrometrically by HPLC–ESI-MS and MALDI-TOF-MS. For HPLC–ESI-MS analysis, culture filtrates of the wild-type *B. amyloliquefaciens* FZB42 and *pks* mutants were extracted with acetonitrile/0.1% formic acid. These extracts were submitted to analytical HPLC coupled with a diode array detector and an ESI-Q Trap mass spectrometer. The HPLC–ESI-MS spectrum of the wild-type *B. amyloliquefaciens* FZB42 extract revealed a number of prominent masses (Table 1), which could be assigned neither to the bacillaenes nor to the difficidins/oxydifficidins nor to any other known metabolite of *B. amyloliquefaciens* FZB42. These mass peaks were found exclusively in the HPLC–ESI-MS analyses of the wild type as well as of the mutants with intact *pks*2 genes (Mln^+; FZB42, CH6, CH8, and CH11) but were absent in the case of mutants CH7, CH12, and CH14 with an inactive *pks*2-operon (Mln^-; Figure 2 and the Supporting Information). Four main compounds (**1**–**4**) were detected by HPLC–ESI-MS at retention times R_t = 6.27, 7.20, 7.39, and 7.43 min, respectively. The molecular masses of the corresponding metabolites are listed in Table 1 [664.2 Da (**1**), 402.3 Da (**2**), 488.3 Da (**3**), and 502.2 Da (**4**)]. The corresponding ion masses $[M+H]^+$ and $[M-H]^-$ were sensitive to collision-induced fragmentations showing identical

Table 1. Macrolactin Production of *B. amyloliquefaciens* FZB42 and Mutant Strains Detected by HPLC–ESI Mass Spectrometry

macrolactin retention time		FZB42		CH 6 Δ*pks*1		CH 8 Δ*pks*3		CH 11 Δ*pks*1*pks*3	
product ions		neg. mode [Da]	pos. mode [Da]	neg. mode [Da]	pos. mode [Da]	neg. mode [Da]	pos. mode [Da]	neg. mode [Da]	pos. mode [Da]
1 at 6.27 min									
$[M-H]^-$	$[M+Na]^+$	663.2	687.2	663.2	687.2	663.2	687.2	663.2	687.2
	$[M+H-H_2O]^+$		647.3		647.3		647.3		647.3
	$[M+H-2H_2O]^+$		629.3		629.3		629.3		629.3
	$[M+H-3H_2O]^+$		611.3		611.3		611.3		611.3
			385.3		385.3		385.3		385.3
			367.3		367.3		367.3		367.3
			349.4		349.4		349.4		349.4
2 at 7.20 min									
$[M+HCO_2]^-$	$[M+Na]^+$	447.3	425.4	447.3	425.4	447.3	425.4	447.3	425.4
$[M-H]^-$	$[M+H-H_2O]^+$	401.3	385.3	401.3	385.3	401.3	385.3	401.3	385.3
$[M-H-H_2O]^-$	$[M+H-2H_2O]^+$	383.3	367.4	383.3	367.4	383.3	367.4	383.3	367.4
	$[M+H-3H_2O]^+$		349.4		349.4		349.4		349.4
3 at 7.39 min									
$[M-H]^-$	$[M+Na]^+$	487.3	511.3	487.3	511.3	487.3	511.3	487.3	511.3
$[M-H-CO_2]^-$		443.2	425.4	443.2	425.4	443.2	425.4	443.2	425.4
		383.3	385.3	383.3	385.3	383.3	385.3	383.3	385.3
		365.3	367.3	365.3	367.3	365.3	367.3	365.3	367.3
			349.3		349.3		349.3		349.3
4 at 7.43 min									
$[M-H]^-$	$[M+Na]^+$	501.2	525.4	501.2	525.4	501.2	525.4	501.2	525.4
			425.3		425.3		425.3		425.3
			385.5		385.5				385.5
			367.4		367.4		367.4		367.4
			349.6		349.6		349.6		349.6

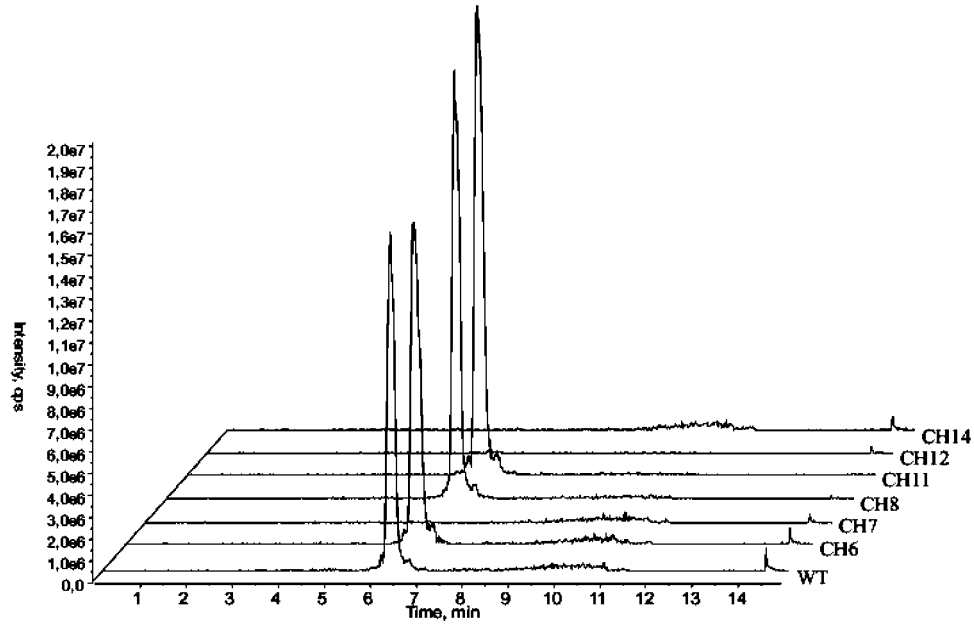

Figure 2. HPLC–ESI-MS chromatograms of the wild-type extract and the extracts of mutants CH6, CH8, and CH11, which are macrolactin producers (Mln^+), and CH7, CH12, and CH14, which are nonproducers (Mln^-). Extracted ion chromatograms of compound **1** are shown.

characteristic fragmentation patterns, indicating they all had the same core structure. Furthermore, similar UV spectra from chromatograms of extracts could be attributed to the corresponding mass peaks of **1**–**4**. From these findings, we concluded that these metabolites belong to the same family of polyketide compounds.

The HPLC–ESI-mass data were found to be consistent with the mass patterns obtained by MALDI-TOF-MS (data not shown). When this technique was applied, *pks*2-specific mass peaks of the protonated forms $[M+H]^+$ and of the alkali adducts $[M+Na, K]^+$ were found for metabolites with molecular masses of 402.3, 488.3, and 664.2, which correspond to compounds **2**, **3**, and **1** detected by LC–ESI-MS, species **1** showing the highest intensities. Also, traces of compound **4** (*m/z* 502.2) were observed. Again, the mass peaks detected for all four species were lacking when *pks*2 was mutated (mutants CH7, CH12, and CH14).

Thus, a group of natural products was sought which exhibit the characteristic mass and UV data found for compounds **1**–**4**. Database and literature searches suggested the macrolactins as promising candidates.[25,26] To verify this assignment, mass spectrometric fragmentation and 2D-NMR studies were performed to elucidate the structures of these compounds. An extract of the culture filtrate of the CH11 mutant was prepared and subjected to high-resolution electrospray ionization–Fourier transform–ion cyclotron resonance mass spectrometry (ESI–FT–ICR-MS). The exact masses for compound **2** and the fragments thereof were determined as 401.23403 Da $[(M-H)^-]$, 383.22273 Da $[(M-H-H_2O)^-]$, and 447.23931 Da $[(M+HCOO^-)^-]$, corresponding to the molecular formulae $C_{24}H_{34}O_5$ $[(M-H)^-_{theor} = 401.23335; \Delta m = 1.695$ ppm], $C_{24}H_{32}O_4$ $[(M-H-H_2O)^-_{theor} = 383.22278; \Delta m = 0.130$ ppm], and $C_{25}H_{36}O_7$ $[(M+HCOO^-)^-_{theor} = 447.23883; \Delta m = 1.073$ ppm]. This results in the molecular formula $C_{24}H_{34}O_5$ for compound **2**, which matches perfectly with the formula for macrolactin A.[20,21]

To confirm this assignment, we isolated compound **2** and determined its structure using 1D and 2D NMR experiments. The resulting NMR data (Supporting Information) were identical to those published in the literature.[27] In this way, compound **2** was identified as macrolactin A, which provides the scaffold for a family of *pks*2-encoded products. According to their molecular masses, the other three compounds were attributed to macrolactin D (**1**), 7-*O*-malonyl-macrolactin A (**3**), and 7-*O*-succinyl macrolactin A (**4**).

After the determination of macrolactin A (**2**) as the biosynthetic product of the *pks*2 gene cluster, we focused on the investigation of the origin of the macrolactin building blocks, the starter unit and the elongation units, specifically. Therefore, feeding experiments were performed with ^{13}C-acetate. Subsequently, ^{13}C-labelled macrolactin A (**2**) was isolated and purified according to procedures established for the unlabelled macrolactins. The interpretation of the ^{13}C-NMR spectrum (Figure 3) of labelled macrolactin A revealed an alternating labelling of the carbon skeleton with ^{13}C. The peaks of 12 ^{13}C-enriched carbon atoms including the starter unit show similar intensities; that is, no significant isotopic dilution was observed, indicating acetate/malonate as the exclusive precursor of the macrolactin skeleton.

In a complementary experiment, 30 *Bacillus* strains, among them 19 *B. amyloliquefaciens* species, 4 *B. subtilis* strains, 2 *B. mojavensis* strains, and 5 *B. licheniformis* strains were tested for their ability to produce the polyketide metabolites macrolactin, bacillaene, and difficidin/oxydifficidin (Table 2). The presence of macrolactin biosynthetic genes (*pks*2 genes) was detected by polymerase-chain reaction (PCR) screening (Figure 4). For this purpose, the primers mln-c1 and mln-c2, derived from the AT domain of the *mlnA* gene of *B. amyloliquefaciens* FZB42, were used. Chromosomal DNA isolated from the macrolactin producer strains including FZB42 yielded a specific 575-bp PCR fragment, whilst the strains unable to produce macrolactin did not show this fragment (Table 2). The genetic ability to synthesize macrolactin was counterchecked by HPLC–ESI-MS analyses. As a result, most of the *B. amyloliquefaciens* strains (14 out of 19) were found to be equipped with the biosynthetic machinery to produce all three polyketide compounds. Another strain of this genus harbored the operons for macrolactin and difficidin formation. The tested *B. subtilis*, *B. mojavensis*, and *B. licheniformis* strains each produced at most only one polyketide compound. *B. subtilis* strains (3 out of 4) and *B. mojavensis* strains (1 out of 2) were bacillaene producers, and the tested *B. licheniformis* strains (3 out of 5) specifically produced difficidin/oxydifficidin. Six strains out of all the investigated *Bacillus* species did not show polyene production and presumably lack all three polyketide gene clusters.

The discovery of three polyketide synthase gene clusters *pks*1–*pks*3 in *B. amyloliquefaciens* FZB42 revealed that this strain has the biosynthetic machinery for the production of at least three

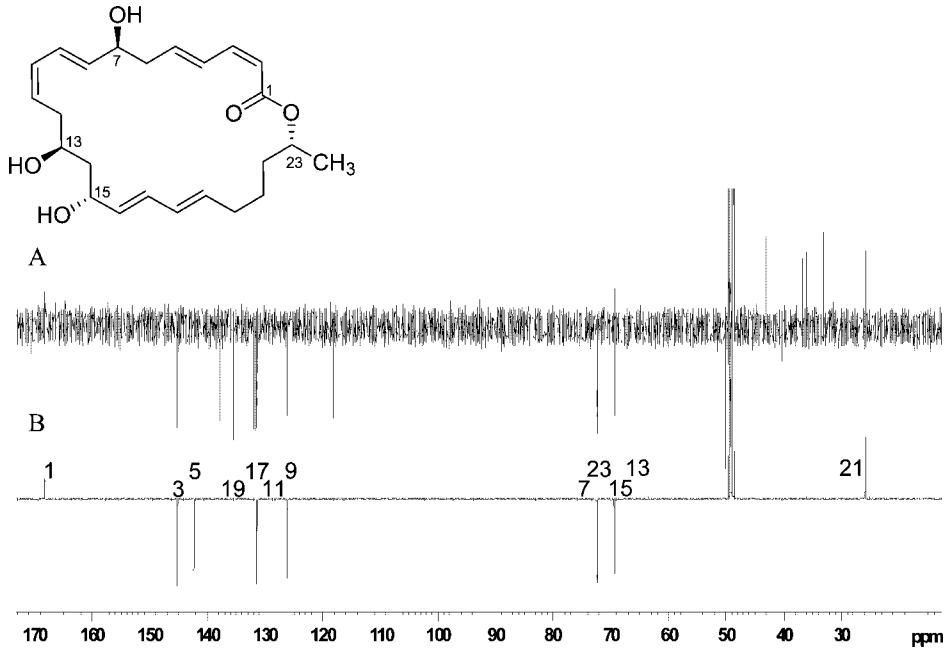

Figure 3. ^{13}C-NMR spectra of (A) macrolactin A (**2**) and (B) assignment of signals of ^{13}C-labelled macrolactin A derived from [1-^{13}C] acetate feeding experiments in MeOH-d_4.

Table 2. Screening of *Bacillus* Strains for Their Production of Polyketides by Polymerase-Chain Reaction (PCR; macrolactin), and HPLC-ESI-MS (MS; bacillaene, difficidin, and macrolactin)

	macrolactin		bacillaene	difficidin
strain	PCR	MS	MS	MS
Bacillus amyloliquefaciens[a] UCMB-5044	+	+	+	+
Bacillus amyloliquefaciens[a] QST713	+	+	+	+
Bacillus amyloliquefaciens[a] FDK21	+	+	+	+
Bacillus amyloliquefaciens[a] FZB42	+	+	+	+
Bacillus amyloliquefaciens[a] B9601-Y2	+	+	+	+
Bacillus amyloliquefaciens[a] UCMB-5113	+	+	+	+
Bacillus amyloliquefaciens[a] FZB 24	+	+	+	+
Bacillus amyloliquefaciens[a] FZB 113	+	+	+	+
Bacillus amyloliquefaciens[a] FZB 111	+	+	+	+
Bacillus amyloliquefaciens[a] GB 03	+	+	+	+
Bacillus amyloliquefaciens[a] FZB 45	+	+	+	+
Bacillus amyloliquefaciens[a] B2	+	+	+	+
Bacillus amyloliquefaciens[a] B3	+	+	+	+
Bacillus amyloliquefaciens[a] 55	+	+	+	+
Bacillus amyloliquefaciens[a] 85	+	+	+	+
Bacillus amyloliquefaciens[a] FZB 109 (A1/3)	+	+	−	+
Bacillus amyloliquefaciens DSM7[b]	−	−	−	−
Bacillus amyloliquefaciens INR-7	−	−	−	−
Bacillus amyloliquefaciens BE 20–2	−	−	−	−
Bacillus subtilis subtilis INR 937	−	−	+	−
Bacillus subtilis subtilis FZB 37	−	−	+	−
Bacillus subtilis spizenii UCMB-5014	−	−	+	−
Bacillus subtilis spizenii AT3	−	−	−	−
Bacillus mojavensis UCMB-5075	−	−	−	−
Bacillus mojavensis MB2	−	−	+	−
Bacillus licheniformis S175 (NCIMB 7724)	−	−	−	−
Bacillus licheniformis ATCC 9789 (B27)	−	−	−	−
Bacillus licheniformis S167 (9945A)	−	−	−	+
Bacillus licheniformis S173 (Gibson 1158)	−	−	−	+
Bacillus licheniformis (B37)	−	−	−	+

[a] Plant associated ecomorph related but not identical with *B. amyloliquefaciens* type strain DSM7 (Reva et al. 2004). Representatives of this group are used in agrobiotechnology for plant growth promotion and biocontrol. [b] Type strain for *B. amyloliquefaciens*.

different kinds of polyketides.[15] Bacillaene and difficidin have been identified as the products encoded by the *pks*1 and *pks*3 operons; however, the *pks*2-specific metabolite remained hitherto unknown. Therefore, our research was focussed on the isolation and identification of this compound. HPLC–ESI-MS and MALDI-TOF-MS analysis of the culture filtrates of the wild-type *B. amyloliquefaciens* FZB42 and of mutant strains bearing mutations in the *pks* operons revealed four *pks*2-specific products. A database and literature search, based on a combination of mass-spectrometric and UV/vis data, suggested that these four *pks*2-specific compounds could be attributed to four variants of the well-known macrolactin family, previously characterized by several authors.[20,21,27] This conclusion was confirmed by the isolation and structure elucidation of macrolactin A (**2**) using mass spectrometric fragmentation studies

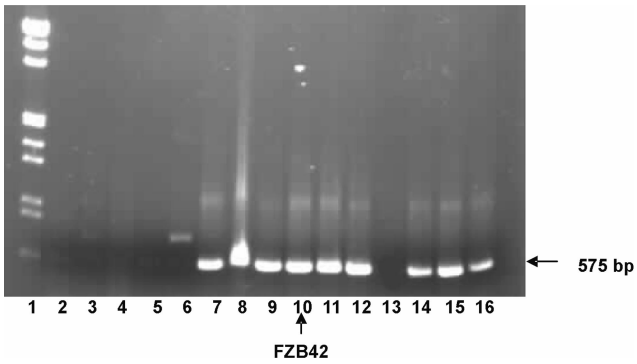

Figure 4. Specific PCR fragments amplified by mln-c1 and mln-c2 derived from the AT domain of the *mlnA* gene product of FZB42. Lane 1: λ-DNA/*Eco*RI+*Hin*dIII marker. Lanes 2–16: (2) *B. amyloliquefaciens* BE 20-2, (3) *B. subtilis subtilis* INR 937, (4) *B. subtilis subtilis* FZB 37, (5) *B. subtilis spizenii* UCMB-5014, (6) *B. subtilis spizenii* AT3, (7) *B. amyloliquefaciens* UCMB-5044, (8) *B. amyloliquefaciens* QST713, (9) *B. amyloliquefaciens* FDK21, (10) *B. amyloliquefaciens* FZB42, (11) *B. amyloliquefaciens* B9601-Y2, (12) *B. amyloliquefaciens* UCMB-5113, (13) *B. amyloliquefaciens* INR-7, (14) *B. amyloliquefaciens* FZB 24, (15) *B. amyloliquefaciens* FZB 113, and (16) *B. amyloliquefaciens* FZB 111.

and 2D-NMR analysis.[27] The other detected macrolactin species are macrolactin D (**1**) as well as 7-*O*-malonyl-macrolactin A (**3**) and 7-*O*-succinyl macrolactin A (**4**).[20,22,23] On the basis of these findings, the *pks*2 operon was renamed as the macrolactin (*mln*) gene cluster.

The *mln* gene cluster exhibits the following characteristic features: Like *pks*1 and *pks*3, *pks*2 is also a modular PKS system of type I.[28] All three operons exhibit a *trans*-AT-architecture (AT = acyltransferase), as has recently been reported for a few other polyketide synthases[29–34] which lack an AT domain in all PKS modules. The lack of AT domains is complemented by acyltransferases encoded on isolated genes acting in trans. Accordingly, a discrete AT gene was detected on *mlnA* of the *mln* gene cluster (Figure 5) upstream of the first PKS module on *mlnB*. Apparently, this discrete enzyme is used iteratively in trans in all elongation steps of the assembly of the growing macrolactin chain to load malonyl-coenzyme A onto the PKS modules of macrolactin synthases MlnB to MlnH.

An unusual feature of the *mln* gene cluster is the presence of "split" modules at the interface of two separate synthase proteins, as has been recently observed in the *pks*-gene clusters for the biosynthesis of difficidin[15] and bacillaene[15,19] as well as pederin[32,33] in bacilli and of chivosazol[34] in the myxobacterium *Sorangium cellulosum*, for example.

The structure of macrolactin A (**2**) has been correlated with the gene organization of the *mln* operon which comprises nine genes *mlnA–I*, as shown in Figure 5. In this way, a model for macrolactin biosynthesis can be proposed (Figure 5) which accounts for most of the functional units necessary to synthesize macrolactin. The scheme for this process is consistent with the ^{13}C-acetate labelling of macrolactin A shown in Figure 5. Feeding experiments imply that acetate/malonate is the only type of building block used in the biosynthesis of this polyketide backbone. Accordingly, the biosynthetic route starts from acetate and is accomplished by 11 successive Claisen condensations involving 11 malonyl units. Presumably, acetate is not directly used as a starter because an acetate-specific acyltransferase has not been detected in the *mln* operon. It rather seems likely that the biosynthetic route is initiated by the loading of a malonyl residue catalyzed by the MlnA-acyl transferase protein followed by the decarboxylation of malonate providing the acetate starter molecule on the first KS domain of MlnB. The elongation process can be widely rationalized by the colinearity rule with the exception of the following notable deviations which are predicted from the polyketide structure. The comparison of the macrolactin A structure with the polyketide assembly line reveals that an enoylreductase (ER) domain in module 2 and two dehydratase (DH) units in modules 7 and 10 are missing for the macrolactin assembly (Figure 5). These activities presumably are provided by other proteins acting in trans or may be accomplished by the iterative usage of other DH domains in the *pks*2 multienzyme system. In any case, the missing enoylreductase function has to be provided externally, because there is no ER domain found in *pks*2 at all. Such complementation of domains has also been observed for difficidin formation encoded by *pks*3[15] and has been suggested for the biosynthesis of other polyketides, such as chivosazol,[34] chalcomycin,[35] and epothilon,[36] for example. The nature of the additional DH and ER domains required for macrolactin production remains to be clarified.

Another striking and not yet completely understood deviation from the colinearity rule is the occurrence of tandemly duplicated ACP domains in modules MlnB, MlnF, and MlnH in the macrolactin assembly line. In addition, two ACPs (Figure 5, shown in gray) were detected in the *mln* operon, one in MlnD and another one in MlnG, which obviously are inactive, because they lack the active serine residue which serves as the attachment site for the 4′-phosphopantetheine cofactor. Similar features have previously been found in the biosynthetic pathways of bacillaene[15,19] and difficidin[15] in *B. amyloliquefaciens* FZB42 as well as in those of other polyketide compounds, such as the pederins,[32,33] chivosazol,[34] curacin,[37] prodigiosin,[38,39] and mupirocin.[40] First information on the possible role of duplicated and triplicated carrier proteins has been obtained in the case of prodigiosin[38,39] and mupirocin[40] biosynthesis. A detailed deletion analysis of the ACPs in the doublet and triplet cluster in the pathway of the antibiotic mupirocin from *Pseudomonas fluorescens* NCIMB 10586 by Rahman et al.[40] revealed that these duplicated ACPs increase antibiotic production. Their mechanism of action seems to be different and depends specifically on the biosynthetic stage involved. Further studies are needed to clarify the role of duplicated ACPs in macrolactin biosynthesis.

The final step of macrolactin formation is the cyclization of the completed polyketide chain by nucleophilic attack of the terminal hydroxy group on the thioester-activated carbonyl. Subsequently, manifold post-assembly-line modifications of the macrolactin scaffold can take place, such as *O*-methylation at each hydroxy group, attachment of a malonyl or succinyl residue at position 7, or a glycosidation at positions 7 and 15, which lead to the synthesis of **1**, **3**, and **4**. These tailoring reactions are presumably performed by external methyl-, acyl- and glycosyltransferases, since they are not found in the proximity of the *pks*2 cluster performing macrolactin biosynthesis. In particular, the presence of sugars on the macrolactin ring may be required for modulation of the antibiotic activity.

In summary, we have demonstrated that macrolactins are ubiquitously found among *B. amyloliquefaciens* strains but are not present in the type strain of this species DSM7 (Table 2). By assignment of the *pks*2 gene cluster (renamed now as the *mln* cluster) to the biosynthesis of macrolactin A, which is assembled from acetate/malonate precursors, a deeper understanding of polyketide biosynthesis in *Bacillus* is attained. Our work may have an impact on the further exploitation of macrolactins for pharmaceutical and biotechnological applications. In particular, 7-*O*-malonyl macrolactin A attained interest for activity testing against problematic nosocomial infections, that is, vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus*.[41]

Experimental Section

General Experimental Procedures. NMR experiments were recorded on a DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a BBI probehead with z gradients. Liquid

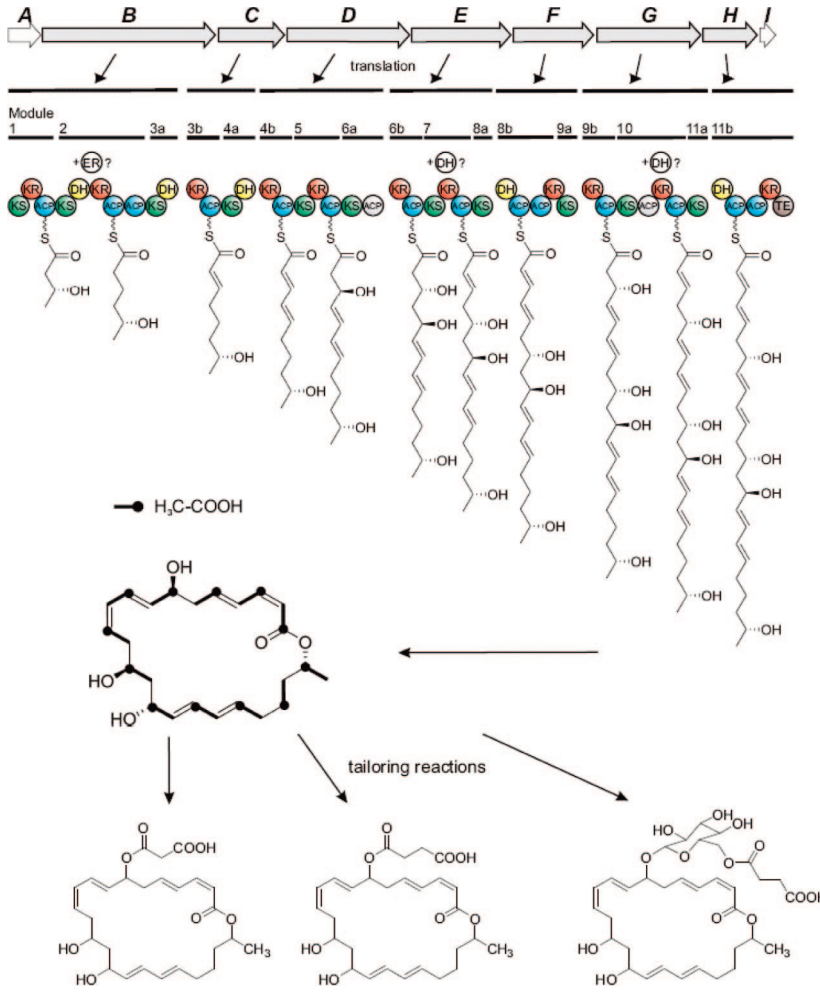

Figure 5. Proposed scheme of macrolactin biosynthesis in *B. amyloliquefaciens* using the type-I polyketide assembly line of *pks*2-encoded MlnB–MlnH. The assembly of ^{13}C-labelled acetate from [1-^{13}C] acetate into the carbon skeleton of macrolactin A is reflected by solid circles. Subsequent tailoring enzymes which are not part of the *pks*2 cluster are postulated to perform the peripheral modification of the macrolactin core structure.

chromatography–mass spectrometry experiments were performed on an Applied Biosystems QTrap 2000 (Applied Biosystems, Darmstadt, Germany) coupled to an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) using a Luna 5 μ C18 column (100 A, 100 × 4.6 mm, Phenomenex, Aschaffenburg, Germany) or a Luna 3 μ C18 column (100 A, 50 × 1.0 mm, Phenomenex, Aschaffenburg, Germany) and a gradient of solvents A (0.1% HCOOH) and B (CH_3CN + 0.1% HCOOH; gradient 0% B to 100% B in 10 min) with a flow rate of 1.5 mL/min or 60 μL/min, respectively. ESI-MS spectra were acquired in the positive and negative modes with a turboionspray voltage, curtain gas, turbo temperature, and nebulizer gas of −4500 V, 30 psi, 300 °C, and 70 psi (negative mode, flow rate at 60 μL/min); of −4500 V, 30 psi, 500 °C, and 50 psi (negative mode, flow rate at 1.5 mL/min); and of 4500 V, 30 psi, 500 °C, and 50 psi (positive mode, flow rate at 1.5 mL/min), respectively. ESI-FT-ICR mass spectra were recorded on an APEX II FTICR mass spectrometer (4.7 T, Bruker-Daltonics, Bremen, Germany). MALDI-TOF mass spectra were recorded with a Bruker Daltonics Reflex MALDI-TOF instrument which was equipped with a 337 nm nitrogen laser for desorption and ionization. A total of 100–200 single scans were accumulated for every spectrum. α-Cyano-4-hydroxycinnamic acid was used as the matrix. For mass spectrometric analysis, lyophilized culture filtrates of *B. amyloliquefaciens* FZB42 and *pks* mutants prepared thereof were extracted with 70% CH_3CN/0.1 trifluoroacetic acid. Portions of the extracts (1–2 μL) were mixed with an equal volume of matrix solution spotted onto the target and air-dried. Positive-ion detection and the reflector mode were used. The acceleration and reflector voltages were 20 and 23.4 kV, respectively, in the pulsed ion extraction mode. A mass gate of 300 Da improved the measurements by filtering out most matrix ions.

Strains, Growth Conditions and PCR Amplification. The strains used in this study and listed in Table 2 are of the following origin: IN937 and INR7 (Joseph Kloepper, Auburn, AL); MB2, FDK21, FZB24, FZB37, FZB42, FZB45, FZB109, FZB111, and FZB113 (Helmut Junge, Abitep GmbH, Berlin, Germany); GB03 (Kodiak, Gustafson Biologicals, Plano, TX); QST713 (Serenade, Agraquest Inc., Davis, CA); RUS1 (isolate from Baksib F, Siberia, Russia); At3, U-5036, U-5017, and all other UCMB strains listed in Table 2 (Oleg Reva, UCM, Ukranian Collection of Microorganisms, Kyiv, Ukraine); strains B2, B3, B55, and B85 (Xuewen Gao, Nanjing Agricultural University, China); B9601-Y2 (Yueqiu He, Kunming, China); and B. *licheniformis* ATCC9789, S167, and S173 (Fergus Priest, Edinburgh, Great Britain). Other strains, such as *B. amyloliquefaciens* BE20-2 and *B. licheniformis* B37, were from our own laboratory stock. For polyketide production, the bacteria were grown in a Landy medium.[7] In order to obtain a high uptake of 1-^{13}C acetate, the Landy medium was modified by using a low concentration of glucose (0.25%). Mutant strain CH11 *(Δbae, Δdif)*[15] was used to produce macrolactin without the other polyketides bacillaene and difficidin. The preculture was grown in Luria broth at 28 °C and 180 rpm overnight and used to inoculate the main culture with a dilution of 1:100. The culture was shaken at 180 rpm at a temperature of 28 °C. A total of 10 mg/mL of 1-^{13}C sodium acetate was added every hour from the 7th to the 12th hour. The supernatant was harvested after a further 12 h of cultivation.

Specific primers used to amplify the AT domain were mln-c1, 5′-ATGCTGTTGCAGGACATAGTC, and mln-c2, 5′-TAGTCAGAATGTTTCCAGGACC. The conditions used for the isolation of chromosomal DNA and PCR amplification were as described previously.[15]

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Supporting Information Available: NMR data of macrolactin A isolated from the CH11 mutant of *B. amyloliquefaciens* FZB42 compared to the NMR data set from the literature; HPLC-DAD–ESI-MS chromatogram of the wild-type extract, macrolactin D (**1**) with a retention time of 6.27 min; HPLC-DAD–ESI-MS chromatogram of the CH7 (Mln^{-}). This material is available free of charge via the Internet at http://pubs.acs.org.

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