

More than Anticipated – Production of Antibiotics and Other Secondary Metabolites by *Bacillus amyloliquefaciens* FZB42

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Key Words

Bacillus amyloliquefaciens · Cyclic lipopeptides · Polyketides · Genome · Bacilysin · Siderophore · Difficidin · Bacillaene · Macrolactin

Abstract

The genome of environmental *Bacillus amyloliquefaciens* FZB42 harbors numerous gene clusters involved in synthesis of antifungal and antibacterial acting secondary metabolites. Five gene clusters, *srf*, *bmy*, *fen*, *nrs*, *dhb*, covering altogether 137 kb, direct non-ribosomal synthesis of the cyclic lipopeptides surfactin, bacillomycin, fengycin, an unknown peptide, and the iron siderophore bacillibactin. Bacillomycin and fengycin were shown to act against phytopathogenic fungi in a synergistic manner. Three gene clusters, *mln*, *bae*, and *dif*, with a total length of 199 kb were shown to direct synthesis of the antibacterial acting polyketides macrolactin, bacillaene, and difficidin. Both, non-ribosomal synthesis of cyclic lipopeptides and synthesis of polyketides are dependent on the presence of a functional *sfp* gene product, 4'-phosphopantetheinyl transferase, as evidenced by knock-out mutation of the *sfp* gene resulting in complete absence of all those eight compounds. In addition, here we present evidence that a gene cluster encoding enzymes involved in synthesis and export of the antibacterial acting dipeptide bacilysin is also functional in FZB42. In summary, environmental FZB42 devoted about 340 kb, corresponding to 8.5%

of its total genetic capacity, to synthesis of secondary metabolites useful to cope with other competing microorganisms present in the plant rhizosphere.

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Introduction

The plant root-colonizing *Bacillus amyloliquefaciens* strain FZB42 is an environmental strain which is distinguished from the domesticated model organism *Bacillus subtilis* 168 by its ability to stimulate plant growth [Idriss et al., 2002, 2007] and to suppress plant pathogenic organisms. Preliminary analysis of the genome sequence revealed the presence of numerous gene clusters involved in synthesis of non-ribosomally produced cyclic lipopeptides [Koumoutsi et al., 2004] and polyketides [Chen et al., 2006] with distinguished antimicrobial action.

In light of the whole genome sequence information [Chen et al., 2007], we will summarize here the impressive capability of *B. amyloliquefaciens* FZB42 to produce a vast array of secondary metabolites aimed to suppress competitive bacteria and fungi within the plant rhizosphere. Interestingly, five of the nine giant gene clusters involved in non-ribosomal synthesis of secondary metabolites are located in close vicinity of the *ter* site in which the bidirectional replication fork meets together (fig. 1).

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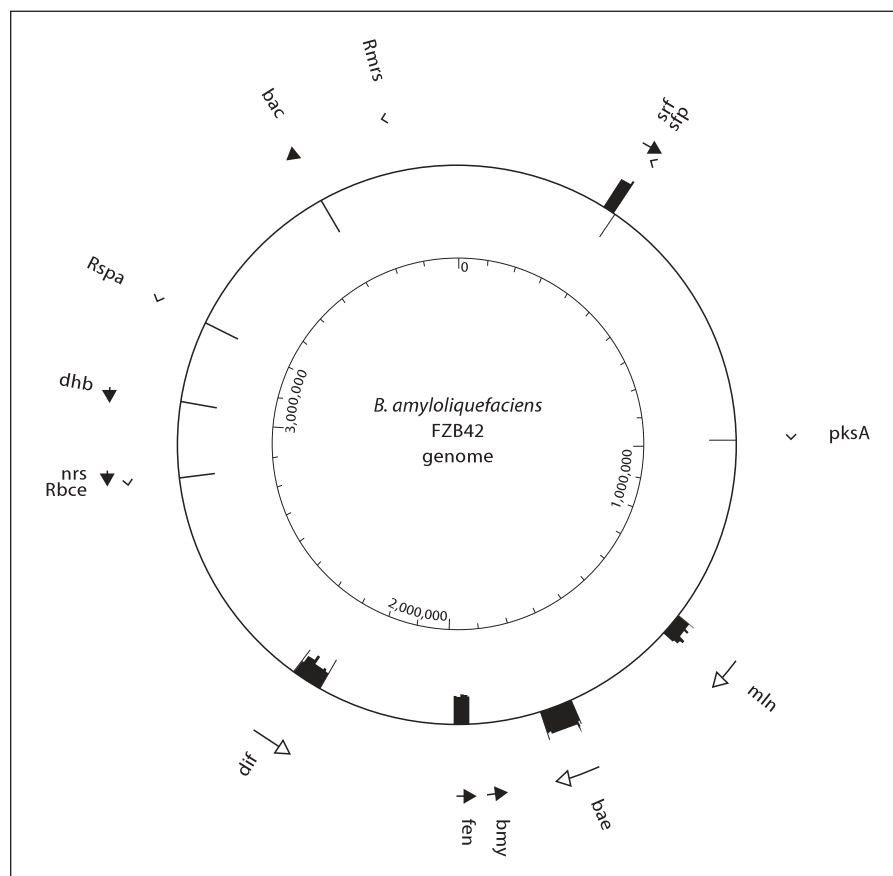
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Fig. 1. Position of genes and gene clusters involved in non-ribosomal synthesis and export of antimicrobial secondary metabolites at the circular map of the *B. amyloliquefaciens* FZB42 genome. The filled arrows at the outermost circle represent the gene clusters for non-ribosomal synthesized peptides surfactin (*srf*), bacillomycin D (*bmy*), fengycin (*fen*), unknown peptide (*nrs*), siderophore bacillibactin (*dhb*), and bacilysin (*bac*) according to their direction of synthesis. The open arrows represent gene clusters involved in polyketide biosynthesis macrolactin (*mln*), bacillaene (*bae*), and diffidicin (*dif*). The open triangles indicate single genes or operons involved in regulation (*pksA*), posttranslational modification (*sfp*), export and resistance mechanisms to detoxify the antibiotics bacitracin (*Rbce*), subtilin (*Rspa*), and mersacidin (*Rmrs*). The degree of similarity to their corresponding genes in *B. subtilis* is also shown. The innermost circle indicates scale of the genome (in bps).



Non-Ribosomally Synthesized Peptides

In spite of their structural heterogeneity, the non-ribosomal peptide antibiotics share a common mode of synthesis, the multicarrier thiotemplate mechanism [Stein et al., 1996]. They are biosynthesized by multimodular proteins termed non-ribosomal peptide synthetases (NRPS). Each elongation cycle in non-ribosomal peptide biosynthesis needs the cooperation of three basic domains. (1) A domain (adenylation domain) selects its cognate amino acid and generates an enzymatically stabilized aminoacyl adenylation. This mechanism resembles the aminoacylation of tRNA synthetases during ribosomal peptide biosynthesis. (2) PCP domain (peptidyl carrier domain) is equipped with a 4'-phosphopantetheine (PPant) prosthetic group to which the adenylated amino acid substrate is transferred and bound by a thioester bond. (3) The condensation domain (C) catalyzes formation of a new peptide bond. The linear organization of such core units (1-3) ensures the coordinated elongation of the peptide product. The assembly of the multifunctional pro-

teins of the peptide synthetases involved in non-ribosomal peptide biosynthesis is reflected in its genetic organization following the colinearity rule [Guenzi et al., 1998]. Cyclic lipopeptides as surfactin, fengycin, and iturin-like antibiotics are widely spread in *B. subtilis* and related strains [Stein, 2005]. The domesticated *B. subtilis* 168 contains three gene clusters devoted to non-ribosomal synthesis of surfactin, fengycin (synonymous to plipastatin) and the siderophore bacillibactin, but is deficient in their syntheses due to a frame shift mutation on the *sfp* gene product, which converts PCP domains to their active form [Mootz et al., 2001]. Moreover, also in the presence of a functional copy of the *sfp* gene, expression of fengycin was found severely reduced in *B. subtilis* 168 due to a *degQ* promoter mutation suggesting that synthesis of fengycin and iturin-like compounds is also dependent on the DegQ regulator protein [Tsuge et al., 1999, 2005]. Besides the *srf* (surfactin) and the *fen* (fengycin) gene clusters, an additional operon, *bmy*, responsible for bacillomycin D production, was detected in the genome of FZB42 [Koumoutsi et al., 2004]. A functional *dhb* gene

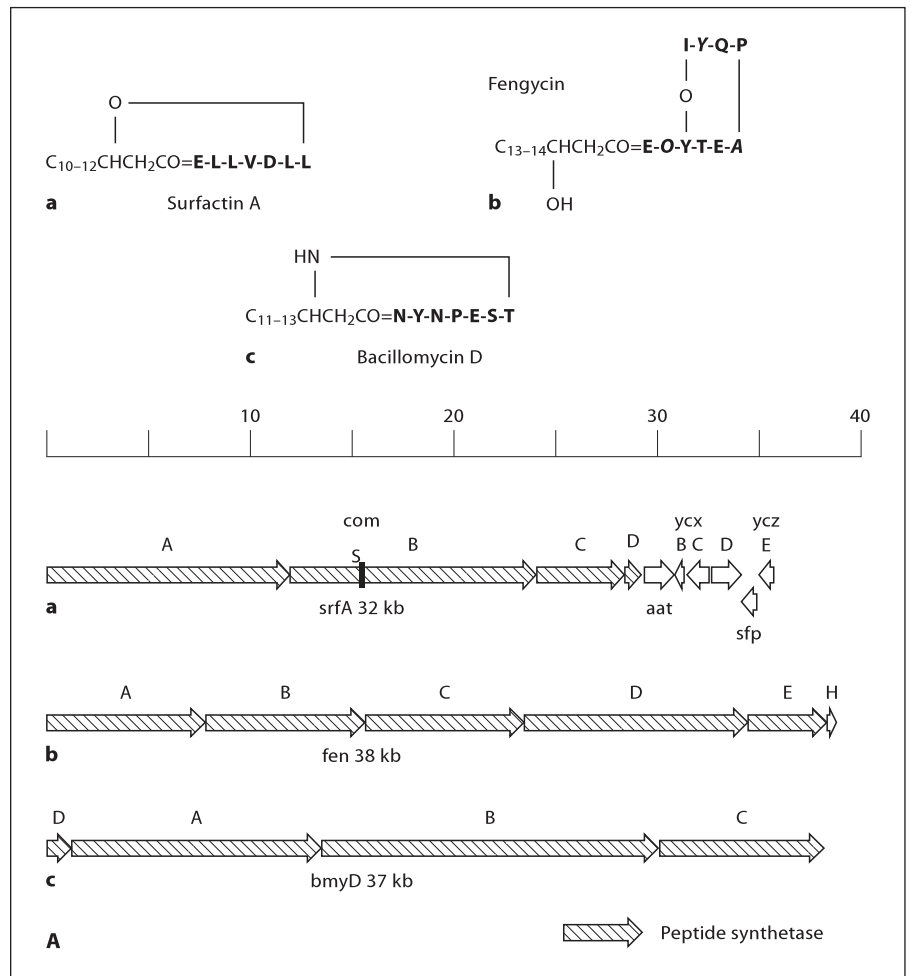


Fig. 2. Structures of secondary metabolites in FZB42 and the giant gene clusters involved in their non-ribosomal synthesis. **A** Lipopeptides surfactin A (**a**), fengycin (**b**), bacillomycin D (**c**).

cluster shown to direct synthesis of the bacillibactin iron siderophore [Vater, unpubl. results] and a further gene cluster, *nrs*, probably directing non-ribosomal synthesis of a hitherto unidentified product also residing in the genome (fig. 1). About 137 kb of the whole genome are devoted to synthesis of such secondary metabolites in FZB42 (fig. 2a).

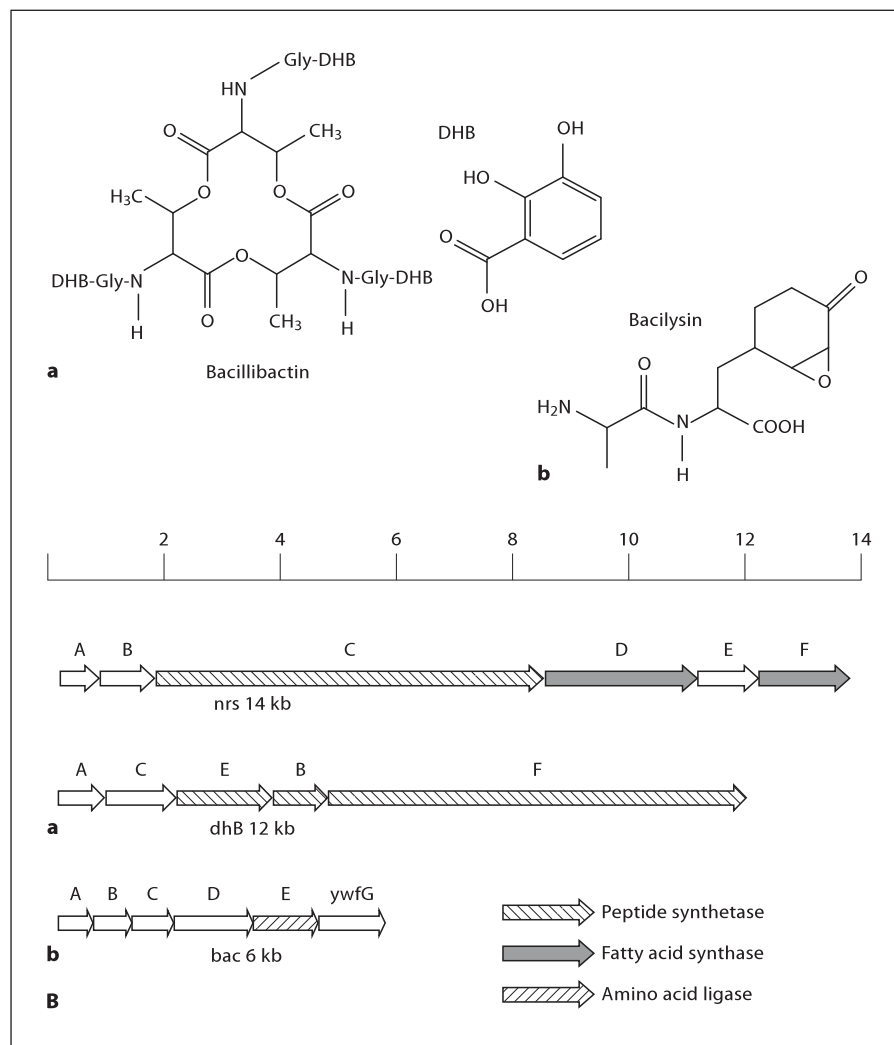
Surfactin

Surfactin is a heptapeptide with an LLDLLDL chiral sequence linked by a β -hydroxy fatty acid consisting of 13–15 carbon atoms to form a cyclic lactone ring structure. Surfactin is surface active and acts hemolytic, antimicrobial and antiviral by altering membrane integrity [Peypoux et al., 1999]. The biological role of surfactin is thought as supporting colonization of surfaces and acquisition of nutrients through their surface-wetting and detergent properties [Neu, 1996]. Mutants of *B. amylo-*

quefaciens, blocked in surfactin biosynthesis, were shown to be severely impaired in biofilm formation [Borriss, unpubl. observations] as previously reported for *B. subtilis* wild-type strains [Hofemeister et al., 2004]. Whilst surfactin is required for formation of aerial structures on the surface of colonies of *B. subtilis*, it simultaneously inhibited development of aerial hyphae and spores by cocultivated *Streptomyces coelicolor* [Straight et al., 2006]. It is likely that surfactin synthesized by *B. amyloliquefaciens* FZB42 protects it against bacteria and enables it to form biofilms, equipping thus the bacterium with powerful antagonistic advantages during surface colonization.

The 26.5-kb *srf* operon present in *B. amyloliquefaciens* FZB42 genome is organized in a similar manner as in *B. subtilis* 168 (fig. 2a). The corresponding genes of these two strains display identity between 72% (*srfAA*) and 83% (*srfAC*) on amino acid level. As in *B. subtilis* 168, the genes present at the left flanking region of the operon are

Fig. 2. Structures of secondary metabolites in FZB42 and the giant gene clusters involved in their non-ribosomal synthesis. **B** Bacillibactin (**a**) and bacilysin (**b**). Synthesis of the dipeptide bacilysin is accomplished by an amino acid ligase reaction (BacD) and does not depend on Sfp. The gene product of the *nrs* gene cluster is still unknown.



hxlBAR. Slight modifications were detected in the region downstream of the *srf* gene cluster. The *B. subtilis* genes *ycxAB* were substituted by two ORFs with unknown function. One of them, *aat*, located adjacent to *srfD* (thioesterase), displays homology to aspartate aminotransferase and has been also detected in biocontrol strain *B. subtilis* B3 [Yao et al., 2003]. No dramatic change in the production of surfactin or of other lipopeptides was observed when *aat* was deleted in strain FZB42. Moreover, the *aat* deletion had no effect on the transcriptional regulation of bacillomycin D [Koumoutsis, 2006].

Bacillomycin D

Bacillomycin D is a member of the iturin family that comprises iturin A, C, D and E, bacillomycin F and L, bacillopeptin and mycosubtilin [Moyne et al., 2004].

Members of the iturin family contain one β -amino fatty acid and seven α -amino acids. The peptide moiety of the iturin lipopeptides contains a tyrosine in the D-configuration at the second amino acid position and two additional D-amino acids at positions 3 and 6. The members of the iturin family exhibit strong antifungal and hemolytic activities and a limited antibacterial activity [Thimon et al., 1995]. In FZB42, bacillomycin D is responsible for the main antifungal activity exerted by this bacterium and was shown to suppress growth of phytopathogenic fungi as *Fusarium oxysporum* [Koumoutsis et al., 2004].

The *bmy* gene cluster is an insertion within the FZB42 genome and separated by only 25 kb from the fengycin gene cluster. It comprised of four genes (*bmyD*, *bmyA*, *bmyB* and *bmyC*) without counterparts in *B. subtilis* 168

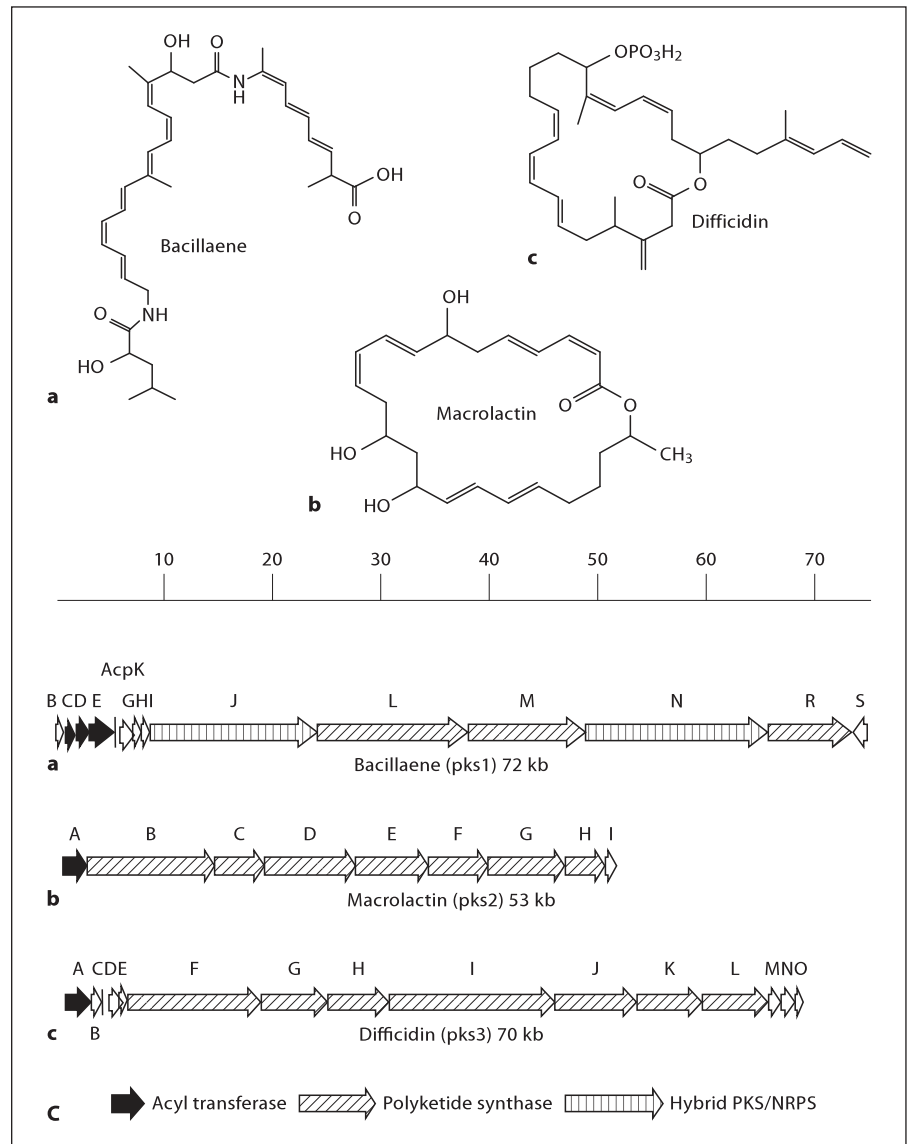


Fig. 2. Structures of secondary metabolites in FZB42 and the giant gene clusters involved in their non-ribosomal synthesis. **C** Polyketides bacillaene (**a**), macrolactin (**b**), and difficidin (**c**).

(fig. 2a). The flanking regions are characterized by DNA rearrangements joining the gene cluster with sequences originally located in different regions of the *B. subtilis* chromosome. In particular, right from the 37-kb cluster, two rearranged gene clusters are situated: *yxjCDEF*, located at position 4,000 kb, and *bioIBDFAW*, located at 3,088–3,094 kb in *B. subtilis*. On the left flanking site, genes located in *B. subtilis* at positions 1,910–1,943 were detected [Koumoutsis et al., 2004]. Notably, the *bmy* operon is inserted at exactly the same position as the iturin A gene cluster in *B. subtilis* RB14 [Tsuge et al., 2001] and the *bmyL* gene cluster in *B. subtilis* A1/3 [Hofemeister et al., 2004].

The first ORF of the *bmy* operon, *bmyD*, encodes a putative malonyl coenzyme A transacylase, similar to FabD, which participates in fatty acid synthesis. *BmyD* is nearly identical to FenF of *B. subtilis* ATCC 6633 and *B. subtilis* RB14. It has been shown that this enzyme is indispensable for iturin production [Tsuge et al., 2001]. The ORFs encoding *BmyA* (3,982 amino acids), *BmyB* (5,633 amino acids), and *BmyC* (2,619 amino acids) are organized like their respective counterparts in the iturin A and mycosubtilin operons. They show strong sequence similarity with those components and consist of an ordered arrangement of domains involved in condensation, adenylation, and thiolation. Seven amino acid-activating mod-

ules can be distinguished: A1, located in BmyA; BmyB1, BmyB2, BmyB3, and BmyB4, located in BmyB, and C1 and C2, located in BmyC. The modules B1, B2, and C1 also contain epimerization domains, directing conversion of amino acids 2, 3, and 6 in a D-configuration. The last domain of this multienzyme system is a thioesterase domain, which is presumably required for release and circularization of the synthesized lipopeptide molecule. Sequence comparison of bacillomycin D with the other iturins showed that sequence variations begin with amino acid IV, although iturin A and mycosubtilin proteins differ only by a reversion at position 6 and 7 [Duitman et al., 1999]. We found that adenylation domains within the first three modules of the bacillomycin D operon shared >97% amino acid identity to the iturin A operon. Homology to the respective domains in the mycosubtilin operon was less pronounced but still >70%. However, homologies were less pronounced for the adenylation domains responsible for activation of the amino acids IV–VII, a finding that corresponds to the variability in the sequence order of the synthesized peptide. The best homology among the last four adenylation domains was obtained between BmyC1, the putative Ser-VI-activating domain, and the corresponding domain of the mycosubtilin operon, which also activates Ser in position 6. The other adenylation domains possibly involved in activating amino acids Pro-IV, Glu-V and Thr-VII, which are unique for bacillomycin D, displayed less homology. Prediction of adenylation domain specificity [Stachelhaus et al., 1999; Challis et al., 2000] revealed that Pro-IV, Glu-V, Ser-VI, and Thr-VII are activated by the adenylation domains in modules B3, B4, C1, and C2, respectively [Koumoutsi et al., 2004].

Fengycin and the nrs Gene Product

Fengycin and the closely related plipastatin are cyclic lipodecapeptides containing a β -hydroxy fatty acid with a side chain length of 16–19 carbon atoms. Four D-amino acids and ornithine (a non-proteinogenic residue) have been identified in the peptide portion of fengycin. Fengycin A is composed of 1 D-Ala, 1 L-Ile, 1 L-Pro, 1 D-allo-Thr, 3 L-Glx, 1 D-Tyr, 1 L-Tyr, 1 D-Orn, whereas in fengycin B the D-Ala is replaced by D-Val. It is specifically active against filamentous fungi and inhibits phospholipase A₂ [Nishikori et al., 1986].

The *fen* five-gene cluster (*fenA-E*) present in FZB42 (fig. 2a) is related to the *pps* operon in *B. subtilis* 168 and is situated at the same locus as this, about 25 kb distant from the *bmy* gene cluster. Because of its similarity to the *fen* gene cluster of the fengycin producers *B. subtilis*

F29-3 [Chen et al., 1995] and *B. subtilis* A1/3 [Steller et al., 1999], the *pps* operon was assigned to fengycin biosynthesis, although *B. subtilis* does not produce this lipopeptide. Interestingly, in the genome of *B. subtilis* ATCC 6633, the mycosubtilin biosynthesis gene cluster devoted to synthesis of an iturin-like compound, is situated at the same location [Duitman et al., 1999], suggesting that additional NRPS operons could be integrated in different ways in this area either as an insertion or as a substitution of existing NRPS operons.

Besides the *srf*, *bmy* and *fen* gene clusters, a fourth gene cluster, *nrs*, encoding a cyclic peptide was detected within the genome of *B. amyloliquefaciens* FZB42 (fig. 1). However, until now, no biological product could be assigned to that sequence, which clearly represents a DNA island within a well-conserved region. The flanking genes *ytzC* (77%) and *ytqA* (92%) are homologous to the respective genes of *B. subtilis*. Low GC content (26–34%) and local deviation of oligonucleotide usage pattern [Reva and Tummler, 2004] underlines island-specific character of this insertion. Within the *nrsABCDEF* gene cluster, a putative NRPS, NrsD, consisting of two modules with Cys-specific adenylation domains was embedded (fig. 2b). Other members of the cluster possessed domains with homology to acyl-CoA-synthetase (*nrsA*), acyl-carrier-protein (*nrsC*), a *mcbC*-like-oxidoreductase (*nrsD*), and thioesterase (*nrsE*).

Biological Activity of FZB42 and Its Lipopeptide-Deficient Mutants

Mutants deficient in synthesis of defined lipopeptides were generated by a gene replacement strategy via double crossover homologous recombination [Koumoutsi et al., 2004]. Biological activity of FZB42 and the respective mutants were assayed in direct growth tests and by bioautography. FZB42 inhibited growth of plant pathogenic fungi as *Fusarium spp.* including *F. oxysporum*, *Gaeumannomyces graminis*, *Rhizoctonia solani*, *Alternaria alternatae*, and *Pythium aphanidermatum* whilst mutant strains deficient in bacillomycin D production were severely impaired in their antifungal activity suggesting that bacillomycin D contributes significantly to its antifungal activity. Mutants bearing knockout mutations within the *srf* and *fen* gene clusters still retained their antifungal activity. Interestingly, fungi grew unaffected in the presence of the double mutant AK3 bearing $\Delta bmyA::Em^R$ and $\Delta fenA::Cm^R$ mutations indicating synergistic action of bacillomycin D and fengycin, against the target microorganism [Koumoutsi et al., 2004]. Our attempts to assign a biological activity to the *nrs* gene product re-

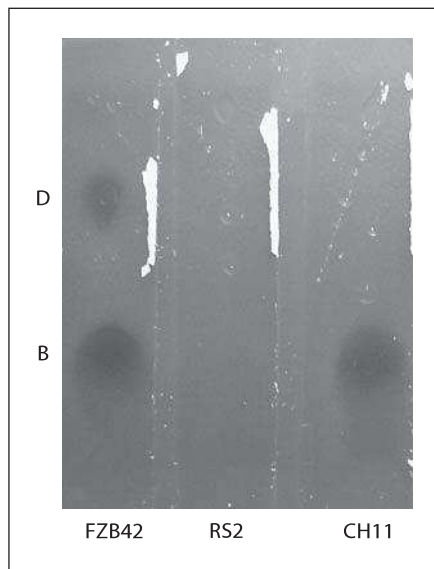


Fig. 3. Bacilysin production in *B. amyloliquefaciens* FZB42 and in mutant strains detected by thin-layer chromatography (TLC) bioautography. Strains FZB42 (wt), RS2 ($\Delta bac::Cm^R \Delta dif::Em^R$) and CH11 ($\Delta bae::Cm^R \Delta dif::Em^R$) were grown in glutamate-free Landy medium for 22 h at 28°C. Supernatants were lyophilized and resuspended in water. TLC was performed in butanol, acetic acid, water (4:1:1). Bioautographs of the TLC plates were performed with *Escherichia coli* K12 as indicator strain as described previously [Chen et al., 2006].

mained hitherto unsuccessful. Mutants with deleted *nrs* gene cluster have no apparent phenotype with respect to antibiotic production or to growth under limited iron concentration [Chen, unpubl. observations].

Bacillibactin

The catecholic iron siderophore bacillibactin, 2,3-dihydroxybenzoyl-Gly-Thr is a cyclic trimeric lactone. Bacillibactin is part of a specific transport system enabling *Bacillus* cells to accumulate and take up limited iron ions from their natural environment. Like other non-ribosomal peptides, its synthesis is dependent on functional Ppant-transferase (Sfp) and therefore bacillibactin is not produced in *B. subtilis* 168, despite the presence of the respective gene cluster [May et al., 2001]. MALDI-TOF MS analysis of the lyophilized supernatant evidenced the production of bacillibactin in FZB42 [Vater, unpubl. data].

The *dhbACEBF* gene cluster within the FZB42 genome was found collinear to the *dhb* gene cluster of *B. subtilis* 168 (fig. 2b). Identities to the respective genes of *B. subtilis* 168 were about 60% for *dhbAC* and 70–80% for *dhbEBF*.

Bacilysin

Bacilysin [L-alanyl-[2,3-epoxycyclohexanone-4]-L-alanine] is a dipeptide antibiotic which contains L-alanine residue at the N-terminus and a non-proteinogenic amino acid, L-anticapsin, at the C-terminus (fig. 2b). The peptide bound with L-alanine proceeds with a non-ribosomal mode catalyzed by an amino acid ligase (bacilysin synthetase). Bacilysin is active against a wide range of bacteria and against yeast *Candida albicans* due to the anticapsin moiety, which becomes released after uptake into susceptible cells. Bacilysin biosynthesis is encompassed by the *bacABCDE* (former *ywfBCDEF*) gene cluster [Steinborn et al., 2005]. The *bacABCDE* gene cluster in FZB42 was collinear to *B. subtilis* 168 (fig. 2b) and its homology ranged between 84 and 93%. Knockout of the *bacA* gene resulted in a complete block of bacilysin production (fig. 3). Contrary to the other non-ribosomal peptides listed above, synthesis of bacilysin was found not to depend on functional Sfp.

Polyketides

Polyketides are a large family of secondary metabolites that include many bioactive compounds with antibacterial, immunosuppressive, antitumor, or other physiologically relevant bioactivities. They are synthesized on modularly organized assembly lines starting from acyl-CoA precursors by decarboxylative Claisen condensations. In general their biosynthetic pathway follows the same logic as in non-ribosomally synthesized peptides and requires at least three domains [Walsh, 2004]. Essential domains of the modules harbored in bacterial type I polyketide synthases are acyl transferase (AT), ketosynthase (KS) and an acyl carrier protein (ACP) which – like PCPs in NRPSs – needs to be activated by Ppant-transferase. The order of the modules dictates the sequence of biosynthetic events. Whilst gene clusters encoding type I polyketide synthases are widespread in different taxonomic groups as *Pseudomonas spp.*, actino-, cyano-, and myxobacteria, the knowledge about *pks* gene clusters in bacilli was until recently restricted to the cryptic *pksX* gene cluster of *B. subtilis* 168. Polyketides difficidin/oxydifficidin, bacillaene [Hofemeister et al., 2004] and macrolactin [Jaruchoktaweechai et al., 2000] were shown as being produced by several wild-type strains belonging to *B. subtilis* and *B. amyloliquefaciens*. We could recently demonstrate that the genome of *B. amyloliquefaciens* FZB42 harbors three giant gene clusters involved in polyketide synthesis [Chen et al., 2006]. Together these gene clusters span nearly 200

kb (fig. 2c). Notably, none of the PKSs encoded by the three *pks* gene clusters harbored modules with cognate AT domains necessary to catalyze an essential step in polyketide synthesis. Instead, one or more genes encoding discrete ATs were detected upstream of the megasynthases encoding genes in all the three *pks* gene clusters of FZB42 [Chen et al., 2006]. This variant of type I polyketide synthase architecture was first described as 'AT-less' type I PKS or 'trans-AT' in a gene cluster involved in pederin biosynthesis [Piel, 2002] and seem to be more abundant than previously anticipated [Shen, 2003]. Discrete ATs act iteratively by loading malonyl CoA onto all PKS modules during polyketide synthesis [Cheng et al., 2003]. Using a combined genetic and chemical approach, we assigned the FZB42 *pks* gene clusters to all the polyketides presently known as being produced by different *Bacillus* strains [Chen et al., 2006, Schneider et al., 2007]. Another unusual feature detected in all three *pks* gene clusters are 'splitted' modules in which domains of one module are located on different proteins [Chen et al., 2006; Butcher et al., 2007].

Bacillaene and Dihydrobacillaene

The *bae* gene cluster was assigned to synthesis of the bacillaene polyketide [Chen et al., 2006], an inhibitor of prokaryotic protein synthesis with the empiric formula $C_{35}H_{48}O_7$ [Patel et al., 1995]. Organization and genomic localization of the *bae* gene cluster is very similar to that of the *pksX* gene cluster of *B. subtilis* 168, but a *pksA*-like sequence corresponding to a putative transcriptional regulator is missing in the operon. Interestingly, a putative regulator *pksR*, a homolog of *B. subtilis* *pksA*, was detected as a discrete gene in a region far upstream from *bae* (fig. 1). Using strain OKB105, a derivative of *B. subtilis* 168 harboring an intact copy of *sfp* encoding the Ppant-transferase, we assigned bacillaene as the synthesis product of *pksX* gene cluster in *B. subtilis* 168 [Chen et al., 2006].

The *bae* gene cluster contained two modules devoted to amino acid synthesis present in the two hybrid NRPS/PKSs indicating that two amino acids are constituents of the bacillaene structure. One of them is glycine, as deduced from the molecular code of the respective adenylation domain located within Bae], the other one can be assigned as alanine according to in vitro experiments performed with the BaeN homolog from *B. subtilis* [Dorrestein et al., 2006]. The presence of three discrete ATs BaeC, D, and E in the upstream region of the *bae* operon is another distinguished trait. Fourier-transform mass spectrometry demonstrated that the BaeC homolog from *B. subtilis* 168 acts as Mal-loading AT [Calderone et al.,

2006]. Recently, it was demonstrated that the hybrid polyketide/non-ribosomal peptide synthase is involved in synthesis of a series of extremely labile, open-chained isomers with bacillaene and dihydrobacillaene as the most abundant representatives [Butcher et al., 2007] (fig. 2c).

Difficidin and Oxydifficidin

Difficidin/oxydifficidin biosynthesis in FZB42 is encompassed by the of *dif* gene cluster which is without counterpart in the genome of *B. subtilis* 168 [Chen et al., 2006]. Difficidin and oxydifficidin, first detected in culture broth of two *B. subtilis* strains, are highly unsaturated 22-membered macrocyclic polyene lactone phosphate esters with broad-spectrum antibacterial activity [Zimmerman et al., 1987]. Its molecular structure has been resolved [Wilson et al., 1987] and the *dif* gene sequences reflect a reasonable synthesis pathway [Chen et al., 2006]. Unlike *bae*, but corresponding to the third *pks* gene cluster (*mln*), the *dif* gene cluster harbored only one trans-AT domain which presumably acts iteratively in transfer of the acyl moiety. Difficidin represents the main antibacterial activity produced by FZB42 [Chen et al., 2006]. Especially promising due to recent efforts of a European research consortium is its suppressive action against the enterobacterium *Erwinia amylovora*, a devastating plant pathogen causing necrotrophic fire blight disease of apple, pear, and other rosaceous plants [www.bactofruct.org].

Macrolactin

Besides bacillaene and difficidin/oxydifficidin, a third polyketide with macrolid-like structure, macrolactin, was previously reported as being produced by bacilli [Jaruchoktawechai et al., 2000]. Macrolactins, originally detected in an unclassified deep-sea marine bacterium, contain three separate diene structure elements in a 24-membered lactone ring [Gustafson et al., 1989]. Until now, at least 17 macrolactins were distinguished and one of them, 7-O-malonyl macrolactin A, has been recently described as efficient against Gram-positive bacterial pathogens [Romero-Tabarez et al., 2006]. Recent preliminary data from mass spectrometry and UV spectrum obtained from a double mutant strain in which the *dif* and *bae* gene clusters have been knocked out revealed that its remaining weak antibacterial activity can be assigned as macrolactin. Moreover, after inactivation of the gene cluster previously designated as *pks2* [Chen et al., 2006], we identified the *pks2* gene cluster as being responsible for macrolactin synthesis in FZB42 [Schneider et al., 2007].

Table 1. Gene clusters involved in non-ribosomal synthesis of lipopeptides and polyketides in the genomes of *B. amyloliquefaciens* FZB42 and *B. subtilis* 168

Compound	FZB42	Size, kb	<i>B. subtilis</i> 168	Identity
Surfactin	<i>srfABCD, aat,334,ycx,CycxD,sfp,yczE</i>	32.0	<i>srfAA,AB,AC,AD,ycxA,ycxB,ycxC,ycxD</i>	73–83%
BacillomycinD	<i>bmyCBAD</i>	39.7	–	0
Fengycin	<i>fenABCDE</i>	38.2	<i>ppsABCDE</i>	60–65%
Putative peptide	<i>nrsABCDEF</i>	15.0	–	0
Bacillibactin	<i>dhbABCDEF</i>	12.8	<i>dhbABCDEF</i>	60–80%
Bacilysin/anticapsin	<i>bacABCDE,ywfg</i>	6.9	<i>ywfgBCDEFG</i>	84–93%
Macrolactin	<i>mlnABCDEFGH</i>	53.9	–	0
Bacillaene	<i>baeBCDE,acpK,baeGHIJLMNRS</i>	74.3	<i>pksBCDE,acpK,pksGHIJLMNRS</i>	52–83%
Difficidin	<i>difAYXBCDEFGHIJKLM</i>	71.1	–	0

Note that except bacilysin all syntheses are dependent on functional Sfp. Due to a frame shift mutation in *sfp* there is no expression of all five gene clusters present in *B. subtilis* 168 genome.

Sfp and *YczE*

The genes located downstream of the surfactin operon, *sfp* (phosphopantetheinyl transferase (PPTase)) and *yczE* (integral membrane protein with unknown function) were found essential for non-ribosomal synthesis of lipopeptides and for synthesis of polyketides [Chen et al., 2006; Koumoutsis et al., 2007]. As mentioned above, Sfp is a substrate-unspecific PPTase which plays an essential role in priming polyketide synthases, NRPS, and siderophore synthetases by covalently converting serine residues in acyl carrier protein (ACP), peptidyl carrier protein (PCP), or aryl carrier protein (ArCP) domains from inactive apo forms to active holo forms. This occurs by tethering the phosphopantetheinyl moiety of the cosubstrate coenzyme A (CoA) in phosphodiester linkage to the hydroxymethyl side chain of the conserved serine residue in the ACP, ArCP, and PCP domains [Walsh et al., 2001]. The introduction of an intact copy of *sfp* into *B. subtilis* 168 recovered production of the polyketide bacillaene in *B. subtilis* 168 [Chen et al., 2006].

Conclusion and Perspective

In conclusion, the genome of *B. amyloliquefaciens* FZB42 contains nine gene clusters that were assigned to direct non-ribosomal synthesis of five bioactive peptides, three polyketides and one unidentified peptide (table 1). Except synthesis of the dipeptide bacilysin, syntheses of all those compounds were found dependent on functional Ppant-transferase (Sfp). These secondary metabolites exhibit strong antifungal and antibacterial activities and enable the bacterium to survive in its natural environ-

ment. As *B. amyloliquefaciens* FZB42 colonizes the plant roots, it inhibits growth of phytopathogenic bacteria or fungi either by depriving them of the essential iron (through the action of bacillibactin) or by directly inhibiting their growth and/or certain of their developmental processes (through the actions of lipopeptides and polyketides). We must note that antibiotic activity is possibly not the only function of those secondary metabolites. Surfactin is involved in intra- and extracellular signaling [Branda et al., 2001] and enabled biofilm formation by *B. amyloliquefaciens* FZB42. Fengycin has been discussed as an elicitor of defense response in plants infected by bacterial or fungal pathogens [Ongena et al., 2005]. Recently, it has been demonstrated that non-ribosomal synthesis of the polyketide bacillaene is accomplished by a singular enzymatic megacomplex, which is attached at the bacterial membrane and is exceeding the size of ribosomes [Straight et al., 2007]. Further studies will also show whether the products of the other giant gene clusters involved in synthesis of the polyketides and lipopeptides discussed in this review are organized in similar megasynthase complexes as now demonstrated for bacillaene.

Notably, *B. amyloliquefaciens* FZB42 devotes 343 kb, more than 8.5% of its total genetic capacity, to non-ribosomal synthesis of bioactive secondary metabolites as peptides, lipopeptides, polyketides and siderophore. Remarkably, 199 kb are used to synthesize the three antibacterial acting polyketides bacillaene, difficidin/oxidydifficidin and macrolactin. Until recently, the average of a *Bacillus* genome devoted to antibiotic production was assumed as to be 4–5% and synthesis of polyketides was not considered as an important feature of endospore-forming

soil bacteria [Stein, 2005]. Unfortunately, the original Marburg strain on which this calculation based has been 'domesticated' in the laboratory since more than a century and was exposed to X-ray treatment in the mid-1940s [Stein, 2005]. Inability to produce nearly none of the bioactive compounds necessary to compete in its natural environment might be due to its special history but hinders its use for significant environmental studies. For comparison, 30 kinds of secondary metabolite gene clusters were detected in the genome of the high-GC soil bacterium *Streptomyces avermitilis*, which is considered as being the most potent producer of secondary metabolites. Altogether, those gene clusters covered a region corresponding to 6.6% of the whole *S. avermitilis* genome (9.02 Mbp) [Ikeda et al., 2003]. Having in hand the whole genome sequence of an environmental *Bacillus* strain, closely related to the model organism *B. subtilis* 168, we are in the position to use the accumulated knowledge body of *Bacillus* molecular biology to investigate the different aspects of lifestyle, inclusive biofilm formation, from a motile low GC-soil bacterium in its natural environment. *B. amyloliquefaciens* FZB42 represents a large group of plant root-associ-

ated bacilli with commercial importance as agent for biocontrol and biofertilization [Borriss, unpubl. observations]. Due to its genetic amenability – allowing use of the same genetic and molecular tools as previously developed for *B. subtilis* 168 – 'true' environmental FZB42 is an ideal candidate for such studies.

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