

DegU and YczE Positively Regulate the Synthesis of Bacillomycin D by *Bacillus amyloliquefaciens* Strain FZB42^{∇†}

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Environmental strain *Bacillus amyloliquefaciens* FZB42 differs from the domesticated model organism of the same genus, *Bacillus subtilis* 168, in its ability to promote plant growth and suppress plant-pathogenic organisms present in the rhizosphere. This behavior is exerted mainly through the production of several nonribosomal cyclic lipopeptides and polyketides, which exhibit a broad range of action against phytopathogenic bacteria, fungi, and nematodes. Here, we provide evidence that the synthesis of the main antifungal agent of *B. amyloliquefaciens* FZB42, bacillomycin D, is regulated in multiple layers. Expression of the bacillomycin D operon (*bmy*) is dependent on a single σ^A -dependent promoter, P_{bmy} and is favored in its natural host by the small regulatory protein DegQ. The global regulators DegU and ComA are required for the full transcriptional activation of *bmy*. DegU retains a key role since it binds directly to two sites located upstream of the bacillomycin D promoter. Moreover, both DegU and a transmembrane protein of unknown function, YczE, act on a later level of gene expression, exerting their posttranscriptional effects in a hitherto-unknown manner.

Plant growth-promoting *Bacillus amyloliquefaciens* strain FZB42 is applied as a biocontrol agent, as it suppresses soil-borne plant-pathogenic organisms. Its antagonistic function is attributed mainly to its ability to produce several secondary metabolites with antibacterial and antifungal activities (5). We have previously reported that FZB42 is a producer of the antibacterial polyketides bacillaene, difficidin/oxydifficidin (6), and a third polyketide which has been identified recently as macrolactin (5). Three cyclic lipopeptides are also produced by *B. amyloliquefaciens* FZB42 (referred to hereafter as FZB42): surfactin, fengycin, and bacillomycin D. Bacillomycin D has been shown to exert the main antifungal activity (20).

Bacillomycin D belongs to the iturin family of lipopeptides, with members such as iturin A and mycosubtilin. It is a cyclic heptapeptide with a β -amino fatty acid moiety and is synthesized nonribosomally according to the multicarrier thiotemplate mechanism (40). The *bmy* operon (37.2 kb) directs the biosynthesis of bacillomycin D and consists of four genes (*bmyD*, *bmyA*, *bmyB*, and *bmyC*) without orthologues in *Bacillus subtilis* 168. Several studies have elucidated the physicochemical and biological properties of peptides that belong to the iturin group (3, 25, 34). The chemical principles for the biosynthesis of iturin-like compounds are also largely characterized (12, 44). In contrast, little is known about the regulatory mechanisms that control the expression of the iturin-like lipopeptides (13).

Nonribosomal peptide synthetases require posttranslational modification to be functionally active (14). In bacilli, this is achieved by the *sfp* gene, which encodes a 4'-phosphopante-

theinyl transferase and posttranslationally converts inactive apoenzyme peptide synthetases to their active holoenzyme forms (14, 18). Strains containing intact synthetases but a dysfunctional *sfp* gene, such as *B. subtilis* 168, which contains intact *srf* and *fen* operons but has a frameshift mutation in the *sfp* gene (27), are unable to produce the antibiotics. However, when *B. subtilis* 168 is complemented with a functional 4'-phosphopantetheinyl transferase, its antibiotic production is restored (30, 45).

It has also been reported that increased expression of the pleiotropic regulator DegQ in *B. subtilis* 168 (1) enhances the antibiotic production (45, 46). Interestingly, most of the natural *Bacillus* isolates that express peptide antibiotics show significantly elevated *degQ* expression compared to that of *B. subtilis* 168, due to the fact that the *degQ* promoter has a more σ^A consensus-like -10 hexamer in those strains (TACACT instead of CACACT) (50). However, whether DegQ directly influences the transcriptional regulation of the antibiotic operons or controls the expression of a posttranscriptional regulator involved in the antibiotic synthesis (for example, Sfp) has not been clarified.

Here, we have developed a genetic tool kit for FZB42 to decipher the complex regulatory network that controls the synthesis of bacillomycin D. 5' deletion analysis of the *bmyD* promoter region and primer extension show that a single σ^A promoter, P_{bmy} , is responsible for the expression of the *bmy* operon, and additional upstream DNA *cis*-acting elements are necessary for the maximal activation of P_{bmy} . DegU is shown to directly bind to two distinct A/T-rich sites within these DNA *cis* elements. Finally, DegU and YczE posttranscriptionally regulate expression of bacillomycin D.

MATERIALS AND METHODS

Strains, plasmids, growth conditions, and DNA transformation. The strains and the plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium and Difco medium 3 (purchased from BD) were used for the cultivation

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>B. subtilis</i>		
MO1099	JH642 MLS ^r amyE::erm trpC2 pheA1	15
AK4	MO1099 amyE::P _{bmyD-342bp} -lacZ (Cm ^r)	This work
AK5	MO1099 amyE::P _{bmyD-125bp} -lacZ (Cm ^r)	This work
AK6	MO1099 amyE::P _{bmyD-62bp} -lacZ (Cm ^r)	This work
AK7	MO1099 amyE::P _{bmyD+29bp} -lacZ (Cm ^r)	This work
AK60	AK4 with pAK64 (Km ^r Phleo ^r)	This work
AK61	AK5 with pAK64 (Km ^r Phleo ^r)	This work
<i>B. amyloliquefaciens</i>		
FZB42	Wild type, producer of lipopeptides and polyketides	19
AK9	FZB42 amyE::P _{bmyD-342bp} -lacZ (Cm ^r)	This work
AK10	FZB42 amyE::P _{bmyD-125bp} -lacZ (Cm ^r)	This work
AK11	FZB42 amyE::P _{bmyD-62bp} -lacZ (Cm ^r)	This work
AK12	FZB42 amyE::P _{bmyD+29bp} -lacZ (Cm ^r)	This work
AK16	FZB42 amyE::P _{bmyD-342bp} -lacZ (Km ^r)	This work
AK17	FZB42 amyE::P _{bmyD-125bp} -lacZ (Km ^r)	This work
AK22	AK16 comA::Em ^r	This work
AK23	AK17 comA::Em ^r	This work
AK26	AK16 yczE::Em ^r	This work
AK27	AK17 yczE::Em ^r	This work
AK32	AK16 degU::Em ^r	This work
AK33	AK17 degU::Em ^r	This work
AK58	FZB42 degU::Em ^r with pAK64 (Km ^r Phleo ^r)	This work
CH4	FZB42 yczE::Em ^r	This work
CH23	FZB42 comA::Em ^r	This work
CH41	FZB42 P _{bmyD400bp} ::P _{spac} -lacI (Cm ^r)	This work
CH42	CH4 yczE::Em ^r P _{bmyD400bp} ::P _{spac} -lacI (Cm ^r)	This work
CH43	TF1 degU::Em ^r P _{bmyD400bp} ::P _{spac} -lacI (Cm ^r)	This work
TF1	FZB42 degU::Em ^r	This work
<i>S. cerevisiae</i>	Indicator strain in agar diffusion assay	Laboratory stock
<i>E. coli</i>		
DH5α	supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA1	Laboratory stock
AK38	DH5α(pREP4,pAK54)	This work
Plasmids		
pDG148	<i>E. coli</i> and <i>B. subtilis</i> shuttle vector, IPTG-inducible P _{spac} promoter, Ap ^r Km ^r Phleo ^r	42
pDG268	pBR322 derivative with promoterless lacZ, integration vector for recombination into <i>B. subtilis</i> amyE, Ap ^r Cm ^r	2
pQE60	Expression vector, IPTG-inducible promoter, His ₆ -Taq, Ap ^r	QIAGEN
pREP4	Repressor plasmid carrying lacI, Km ^r	QIAGEN
pECE73	Cm ^r →Km ^r exchange vector, Ap ^r	41
pECE149	LacI-bearing vector plasmid	BGSC
pUCmR	Exchange of Amp ^r by Cm ^r cassette in pUC18	This work
pAK5	pDG268 carrying a fragment of <i>bmyD</i> from bp -342 to +184 ^a	This work
pAK6	pDG268 carrying a fragment of <i>bmyD</i> from bp -125 to +184	This work
pAK7	pDG268 carrying a fragment of <i>bmyD</i> from bp -62 to +184	This work
pAK8	pDG268 carrying a fragment of <i>bmyD</i> from bp +29 to +184	This work
pAK9	<i>B. amyloliquefaciens</i> FZB42 integration vector amyE::lacZ, Ap ^r Cm ^r , pDG268 derivative	This work
pAK16	pAK9 carrying a fragment of <i>bmyD</i> from bp -342 to +184	This work
pAK17	pAK9 carrying a fragment of <i>bmyD</i> from bp -125 to +184	This work
pAK18	pAK9 carrying a fragment of <i>bmyD</i> from bp -62 to +184	This work
pAK19	pAK9 carrying a fragment of <i>bmyD</i> from bp +29 to +184	This work
pAK54	pQE60 carrying degU of <i>B. amyloliquefaciens</i> FZB42	This work
pAK64	pDG148 carrying a 208-bp fragment of degQ	This work
pCH41A	pUCmR carrying lacI (Cm ^r)	This work
pCH41B	pCH41A lacI P _{spac} bmyD'' (Cm ^r)	This work
pCH41C	PCH41B bmyD' lacI P _{spac} bmyD'' (Cm ^r)	This work

^a The positions are relative to the *bmy* transcriptional start.

of bacteria. For antibiotic production and matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometric (MS) analysis, single colonies of FZB42 and its derivatives were incubated overnight in 10 ml LB with shaking (210 rpm) at 37°C. Landy medium (23) was inoculated with 1% of FZB42 and cultivated at 37°C and 210 rpm. At an optical density at 600 nm of 0.4 to 0.5, 0.6 mM IPTG (isopropyl- β -D-thiogalactopyranoside) was added. After a further 36 h of shaking, the supernatant was gathered for MALDI-TOF measurements. Thirty microliters of supernatant was used per hole for the agar diffusion inhibition test, using a growing culture of *Saccharomyces cerevisiae* as the indicator strain.

DNA transformation in *B. subtilis* MO1099 was performed as described previously (38). The media and buffers used for DNA transformation in FZB42 were the same as those described by Kunst and Rapoport (22), whereas competent cells were prepared using a previously described protocol (19, 20), which is based on the fact that FZB42 is competent in late exponential phase.

Primer extension analysis. Total RNA was isolated from FZB42 cells grown in Difco medium, using the Nucleo Spin RNA L kit (Macherey Nagel). For the primer extension analysis, 40 μ g of total RNA was mixed with 0.15 μ M 5'- γ -³²P-labeled primer (see Table S1 in the supplemental material for the two primers used in this study) at 70°C for 5 min. Then, 4 μ l 5 \times reverse transcriptase buffer, 2 μ l deoxynucleoside triphosphates (10 mM each), 1 μ l RNase inhibitor (40 units), and 1 μ l reverse transcriptase (200 units; Fermentas) were added to the mixture, and incubation was continued for 1 h at 42°C. The reaction was stopped by adding loading buffer. The samples were subsequently separated on a denaturing polyacrylamide sequencing gel (8 M urea, 7% acrylamide). Dideoxynucleotide sequencing reactions were done using the same 5'- γ -³²P-labeled primer and the Thermo Sequenase cycle sequencing kit (USB) according to the manufacturer's instructions.

Construction of *bmyD::lacZ* transcriptional fusions in *B. subtilis* MO1099 and *B. amyloliquefaciens* FZB42. Vector pAK9 (Table 1) is derived from the *B. subtilis* 168 integration vector pDG268 (2) and was constructed to enable us to integrate single-copy transcriptional fusions at the *amyE* locus in the FZB42 chromosome via double-crossover recombination. The highly homologous (89% on the nucleotide level) 5' end of the *B. subtilis amyE* gene in pDG268 was kept intact, whereas the less homologous (81%) 3' end of the *B. subtilis amyE* gene in pDG268 was replaced by the corresponding fragment of the *amyE* gene from FZB42.

EcoRI/BamHI DNA fragments of the *bmyD* promoter region of FZB42, consisting of a common downstream end and variable upstream ends, were amplified by PCR using the primers *bmyD1* to *bmyD5* (these and the other primers used are listed in Table S1 in the supplemental material). The fragments were cloned into the *B. subtilis* integration vector pDG268 (2) and into its FZB42 counterpart pAK9, in each case upstream of the promoterless *lacZ* gene (Table 1). The derivative plasmids of pDG268 and pAK9 were linearized with XhoI and transformed into competent cells of *B. subtilis* MO1099 and FZB42, respectively. The correct chromosomal integration of the transcriptional fusions at the *amyE* locus was verified by PCR and Southern hybridization. (For a schematic representation of the fusions obtained, see Fig. 2A.)

Construction of plasmids and generation of *B. amyloliquefaciens* FZB42 mutant strains. The *comaA* mutant strain was constructed by amplifying a 2,092-bp PCR product with primers *coma-1/2* and cloning in pGEM-T. To construct CH23, a central 104-bp fragment was removed from the *comaA* gene with Kspal and Eco72I, and the *Em^r* gene was inserted as a selective marker.

The *lacI* gene was prepared from plasmid ECE149 (BGSC) using restriction enzymes SmaI and BamHI and cloned into the BamHI- and Eco47III-digested plasmid pUCcmr to generate plasmid pCH41A.

The P_{spac} promoter was amplified with primers *pspac-1*-BamHI and *pspac-2* using the plasmid pMUTIN-GFP+ (BGSC) as a template. The 5' part of the *bmyD* gene cluster was amplified with primers *bmyDL-1* and *bmyDL-2*-SacI using FZB42 chromosomal DNA as a template. Both PCR fragments were fused together by the splicing by overlapping extension (SOE) method using *pspac-1*-BamHI and *bmyDL-2*-SacI as additional primers. The SOE-created fragment was digested with SacI and BamHI and inserted into the plasmid pCH41A, yielding pCH41B. To allow double-crossover homologous recombination, the further upstream promoter flanking region was amplified with primers *bmyDR-1*-HindIII and *bmyDR-2* from FZB42 chromosomal DNA and inserted into pCH41B to create pCH41C. Integrative plasmid pCH41C was linearized with ScaI and transformed into FZB42 to create the mutant CH41 strain harboring the IPTG-inducible P_{spac} promoter. Chromosomal DNAs from the mutant strains TF1 (*degU*) and CH4 (*yczE*) were used in transformation of CH41, yielding CH42 and CH43.

To generate plasmid pAK64, a 0.2-kb DNA region encompassing the *degQ* coding region was amplified by PCR using the primer pair *degQ1/2* and subse-

quently cloned into the HindIII/SphI-digested replication vector pDG148 under control of an IPTG-inducible promoter. Plasmid pAK64 was then transformed into AK4, AK5, and TF1, resulting in strains AK60, AK61, and AK58, respectively.

β -Galactosidase assay. Specific β -galactosidase activity was determined from growing liquid cultures in Difco medium as described by Cutting and Van der Horn (7). β -Galactosidase was assayed using *o*-nitrophenyl- β -D-galactopyranoside as the substrate and is reported in Miller units (26).

MALDI-TOF MS analysis. The wild-type and mutant strains were grown in Landy medium for 24 h. Lipopeptide products of FZB42 were identified in extracts of lyophilized culture filtrates by MALDI-TOF MS as previously described (20). α -Cyano-4-hydroxycinnamic acid was used as the matrix.

Purification of DegU. A C-terminal His₆ tag fusion to DegU was constructed using the commercially available plasmid pQE60 (QIAGEN). The *B. amyloliquefaciens* FZB42 *degU* gene was PCR amplified using the primers *degU60a/b* and was further cloned into an NcoI/BglIII-digested pQE60 (QIAGEN) downstream of an IPTG-inducible promoter. The resulting plasmid, pAK54, was cotransformed with pREP4 (QIAGEN) into *Escherichia coli* DH5 α competent cells, yielding AK38. To isolate DegU-His₆, AK38 was grown and cells were harvested as previously described (17). The cells were then lysed and the protein was purified using the Protino Ni-1000 kit, according to the manufacturer's instructions (Macherey Nagel).

Electrophoretic mobility shift assay (EMSA). A 450-bp DNA fragment harboring the *bmyD* promoter region between bp -342 and +108 relative to the translational start was amplified using primers *bmyD1* and 5'- γ -³²P-labeled *rev1*. The fragment was incubated at 37°C for 20 min with different amounts of purified DegU in binding buffer [20 mM Tris-HCl (pH 8), 100 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, 10% glycerol, 0.05% Nonidet P-40, 0.05 mg/ml bovine serum albumin, and 0.05 mg/ml poly(dI-dC) as a competitive nonspecific DNA]. The reaction mixtures were then separated on nondenaturing gels (8% polyacrylamide) in 1 \times Tris-borate-EDTA buffer at 60 V.

DNase I footprinting assay. DNase I footprinting experiments were performed as described previously (48). A DNA fragment carrying the extended version of the *bmyD* promoter (450 bp; obtained by PCR amplification using primers *bmyD1* and *rev1*) or the supercoiled plasmid pAK16 (carrying the same DNA fragment) was incubated in binding buffer with different amounts of DegU protein (0/0.8 and 1.6 μ M) for 20 min at 37°C. Complexes were then treated with DNase I (0.6 μ g/ml; Sigma) for 20 seconds, and the reaction was stopped by addition of nonspecific DNA (salmon sperm; 1.75 ng/ μ l) and rapid chilling on ice. Primer extension followed, with 5'- γ -³²P-labeled *bmyD1* and 5'- γ -³²P-labeled *rev1* for the template strand and for the nontemplate strand, respectively (denaturing temperature, 94°C [30 s]; annealing temperature, 58°C [30 s]; extension temperature, 72°C [30 s] [23 cycles]). The samples were then separated on 8 M urea-7% polyacrylamide sequencing gels and visualized using the Phosphorimager 445SI (Molecular Dynamics).

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers of two contigs containing the surfactin region together with the *yczE* gene (AJ575417) and the bacillomycin D operon (AJ575642) of FZB42 have been published previously (30). The accession numbers of the other gene sequences used in this study are as follows: *degU*, AM494075; and *degQ-comP-comA*, AM494074. They were derived from the complete genome sequence of FZB42, which is available under GenBank accession number CP000560.

RESULTS

Identification of the transcriptional start site of the *bmy* operon. Primer extension analysis revealed two adjacent transcriptional start sites for the *bmy* operon: an adenine (A) nucleotide and a thymine (T) nucleotide, located 58 and 57 bp upstream of the gene's translation initiation codon (Fig. 1A). Sequences resembling the consensus of the -10 and -35 elements of the housekeeping sigma factor σ^A were found upstream of the mapped transcriptional start site (Fig. 1B). -35 (TATACA) and -10 (TAGGAT) hexamers could be identified in the proper distances from the transcriptional start and displayed 4/6 matches to the corresponding consensus sequences of σ^A . The spacer between them is 18 bp long and contains an extended -10 region directly upstream of the -10 hexamer, i.e., CATGc (the underlined nucleotides match the

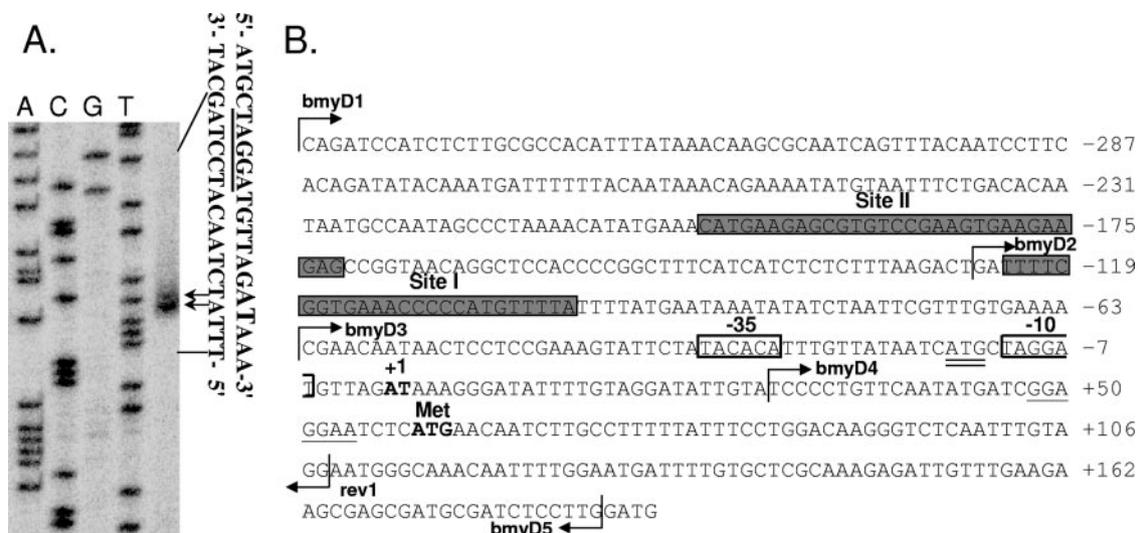


FIG. 1. Mapping of the transcriptional start of the *bmy* operon by primer extension analysis. (A) Primer extension was performed using the 5'-³²P-end-labeled primer *rev1*. The first four lanes result from dideoxynucleotide sequencing reactions. Arrows indicate the positions of the two adjacent *bmyD* transcriptional starts. The respective DNA sequences are shown on the right. The transcriptional starts are indicated by larger letters, whereas the putative -10 element is underlined. (B) The *bmyD* promoter region. The positions of the two adjacent transcriptional starts (boldface, +1), the translational start (boldface, Met), the putative ribosome binding site (underlined), the -35 and -10 hexamers (boxes), and the extended -10 region (double underlined) are indicated. The 5' end sites of the oligonucleotides used for primer extension (*rev1*) and for construction of reporter fusions (*bmyD1* to -*D5*) are marked by arrows. The two DegU binding sites (sites I and II) are shaded.

consensus, TRTGn [49]). Therefore, the promoter of *bmyD* (P_{bmy}) seems to be recognized and utilized by the vegetative sigma factor, despite the fact that *bmy* shows maximal expression during stationary phase (see below). Even though extensive attempts were made to identify additional transcriptional start sites upstream of the P_{bmy} , such sites were not found (data not shown).

DegQ is responsible for differences in *bmy* expression in *B. subtilis* MO1099 and *B. amyloliquefaciens* FZB42. In order to monitor the transcriptional regulation of bacillomycin D, four reporter fusions of the *bmy* promoter region (Fig. 2A) to *lacZ* were generated. A series of nested fragments with a common downstream end (bp +184 relative to the transcriptional start) and variable upstream ends (bp -342, -125, -62, and +29 relative to the transcriptional start) were fused to a promoterless *lacZ* gene in pDG268 and pAK9 (see Materials and Methods). The transcriptional fusions were introduced as single copies in the chromosome of the heterologous host *B. subtilis* MO1099 and the homologous host FZB42, and their expression was determined throughout the growth cycle.

In *B. subtilis* MO1099, all four strains carrying the *bmyD::lacZ* reporter fusions were silent during the logarithmic phase (Fig. 2B). Upon entry into stationary phase, the transcriptional activity of AK4, AK5, and AK6 increased and reached its maximum after a further 3 hours. AK7 remained silent and did not show any β -galactosidase activity, since it lacked the entire promoter. The fact that the fusions of AK6 and AK5 showed no difference in their expression pattern throughout the whole growth cycle suggested that no additional *trans*-activating factor binds to the region between bp -125 and -62. AK4 exhibited slightly but reproducibly higher activity than the other two strains (AK6 and AK5) during

stationary phase, indicating that the region between bp -342 and -125 might carry additional *cis*-activating elements.

In FZB42, the overall expression pattern of the various fusions was similar to that observed in *B. subtilis* MO1099, but with a notable exception (Fig. 2C). The strain carrying the full-length upstream promoter region, AK9, exhibited four- to fivefold more β -galactosidase activity in stationary phase than the strains harboring the shorter fusions (AK10 and AK11). This difference in expression levels between AK9 and AK10/AK11 is considerably higher than that observed for the corresponding strains of *B. subtilis* MO1099 (Fig. 2B), underlining the importance of this DNA upstream region for the full transcriptional activation of the *bmy* operon in its natural host.

Transcription of *degQ* in FZB42 is driven by the stronger σ^A promoter version, whereas *B. subtilis* MO1099, a derivative of the strain 168, carries the "defective" *degQ* promoter version. To test whether DegQ is responsible for the differential *bmy* expression patterns in the two strains, pAK64 (where *degQ* is expressed from an IPTG-inducible promoter [Table 1]) was transformed into AK4 and AK5 (Fig. 2A). As presented in Fig. 2D, the presence of uninduced pAK64 did not alter *bmy* expression from the two strains. Upon induction of pAK64, though, a significant 2.5-fold increase in the activity of AK4 could be observed. Since DegQ is present in higher levels in FZB42 than in *B. subtilis* MO1099, DegQ accounts, at least partially, for the different *bmy* expression in the two host strains. This is the first direct piece of evidence that DegQ controls the transcription of peptide antibiotics and not a subsequent step in their production. In the case of bacillomycin D, this effect seems to be dependent on a far-upstream region of the promoter. Since that DegQ is not a DNA binding protein, the effect on bacillomycin D should be mediated in an indirect

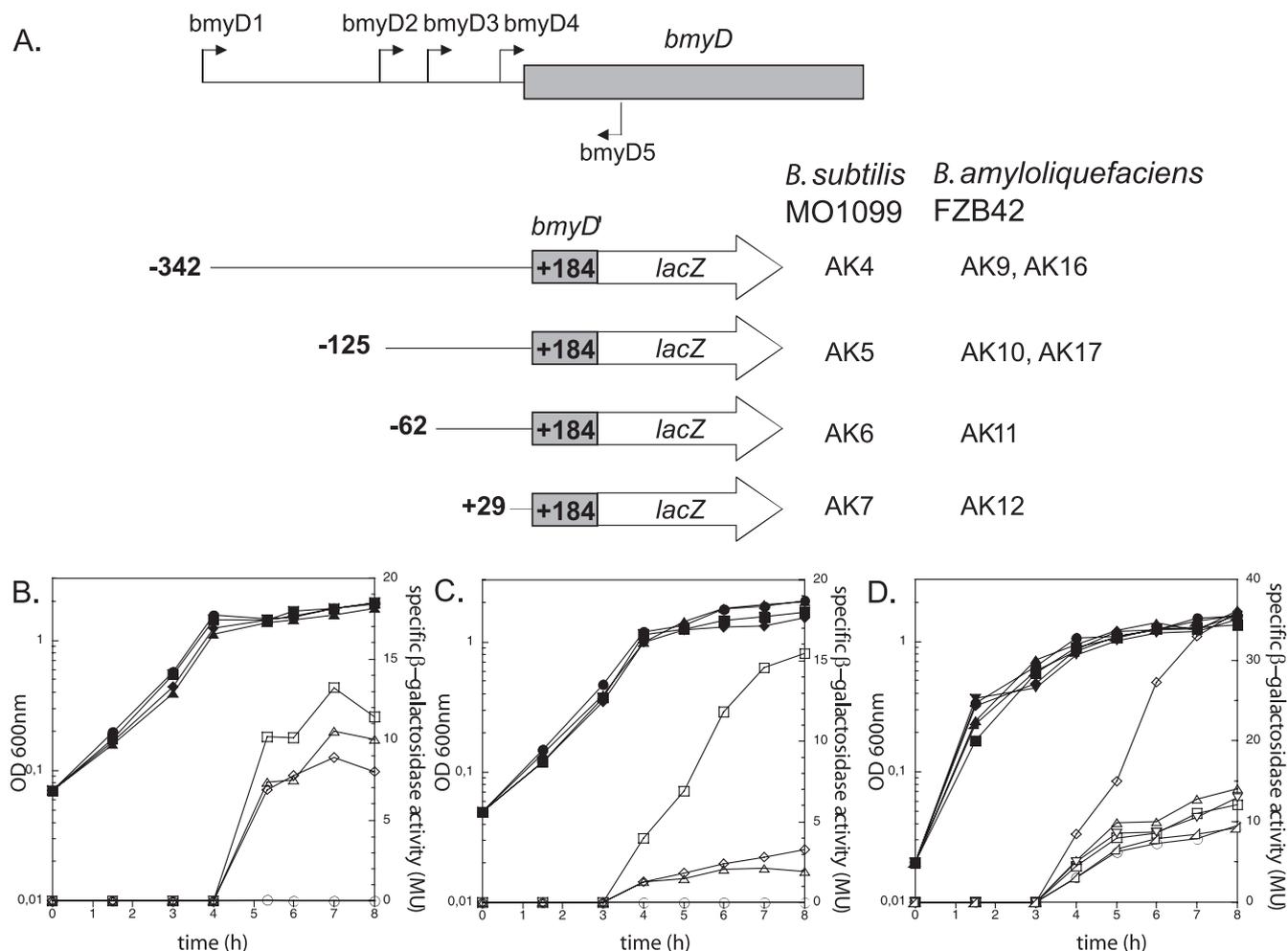


FIG. 2. Mapping of the *bmyD* promoter region by 5' deletions. (A) Schematic representation of the different *bmyD::lacZ* fusions used in this study. The filled arrows indicate the primers used for generating the various reporter fusions (*bmyD1* to *-D5*), whereas the 5' and 3' end termini of the *bmyD::lacZ* fusions are denoted with their nucleotide position relative to the transcriptional start. The derivative *B. subtilis* MO1099 and FZB42 strains that carry the respective fusions are shown on the right (see Table 1). (B and C) Expression of the series of *bmyD::lacZ* fusions, with different 5' end termini, in *B. subtilis* MO1099 (B) and in FZB42 (C). Cells were grown in Difco medium at 37°C, and optical densities (OD) (closed symbols) and β -galactosidase activities (open symbols) were determined during growth. Squares, strains AK4/AK9; diamonds, strains AK5/AK10; triangles, strains AK6/AK11; circles, strains AK7/AK12. (D) Effect of DegQ on the expression patterns of the various *bmyD::lacZ* fusions in *B. subtilis* MO1099. Squares, strain AK4 without plasmid; circles, strain AK5 without plasmid; diamonds, strain AK60 (AK4+pAK64) with plasmid induced; triangles facing down, strain AK60 with plasmid uninduced; triangles facing up, strain AK61 (AK5+pAK64) with plasmid induced; rectangle-triangles, strain AK61 with plasmid uninduced.

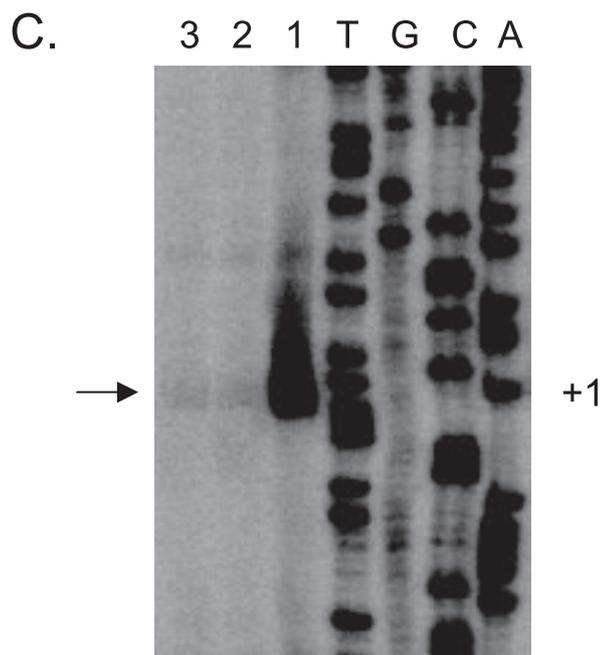
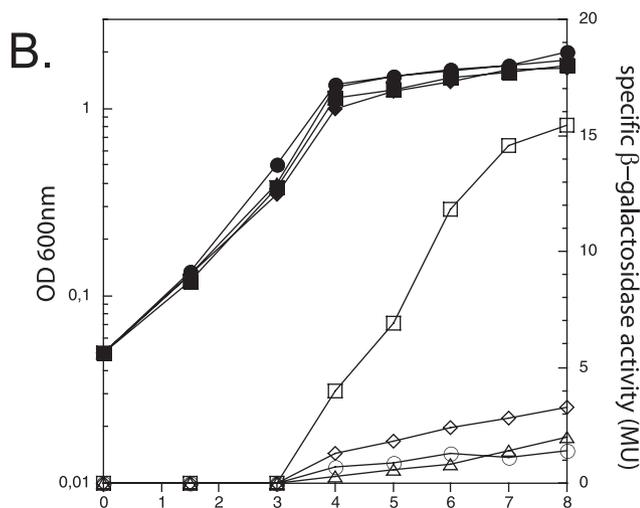
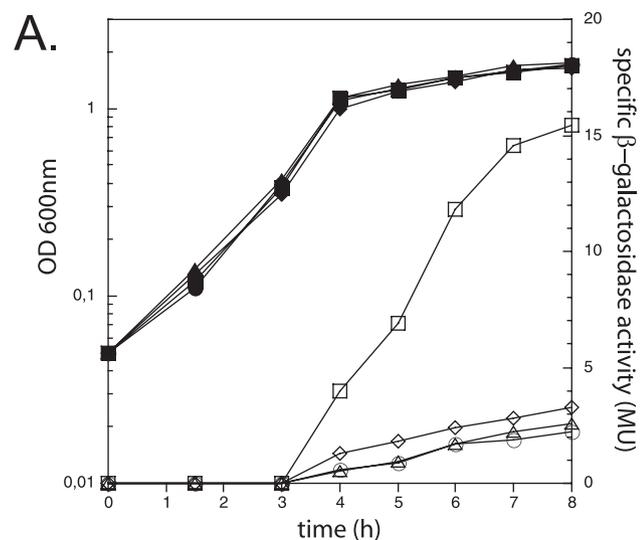
manner, possibly through modulating the activity of another transcriptional regulator(s).

The global transcriptional regulators DegU and ComA control the production of bacillomycin D. In order to obtain further information concerning the transcriptional regulation of bacillomycin D, a series of mutations in transcriptional regulatory proteins of FZB42 were generated. The mutations were then introduced into strains AK9 and AK10 (AK11 was excluded from this analysis, since it displays the same pattern of gene expression as AK10 [Fig. 2C]), and the β -galactosidase activities of the mutant strains were monitored along the growth curve and compared to that of the wild-type strain. Using this approach, several proteins involved in the regulation of the *bmy* operon were identified (Fig. 3A and data not shown).

Two two-component response regulator proteins were found

to be essential for full activation of bacillomycin D expression: DegU, which is known to control the expression of degradative enzymes (21, 28) and to be involved in the initiation of competence (17), and ComA, a regulator of late competence genes (16) and surfactin production (35, 36). Inactivation of the genes coding for each of these proteins resulted in severely impaired promoter activity during stationary phase, especially in strains harboring the whole promoter region (AK9 derivatives). *bmy* transcription was three- to fourfold lower in the *degU* and *comA* mutant derivatives of AK9 than in the parental strain and was similar to that of the wild-type strain AK10, which carries the shorter promoter region. Furthermore, in the absence of DegU or ComA, the activities of AK10 derivatives were also reduced in comparison to that of the parental strain, albeit to a much smaller extent (Fig. 3A and B).

Therefore, DegU and ComA are both required for the full



activation of the *bmyD* promoter, and their effects seem to be exerted mostly through the DNA region located between bp -342 and -125 upstream of the *bmyD* transcriptional start. Note that despite the severity of the reported effects, the transcription of *bmyD* is not completely silenced in any of those mutants, implying that the σ^A -dependent promoter retains basic activity even in the absence of the two regulators.

To verify that the effects of DegU and ComA on transcription of the *bmy* operon were exerted via the unique house-keeping promoter of the identified operon, we performed additional primer extension experiments using wild-type FZB42 and its derivative mutant strains (Fig. 3C). Consistent with the results obtained from the *lacZ* reporter fusions, the two mutant strains exhibited significantly lower *bmy* transcript levels than the parental strain. This finding confirms the fact that cells lacking those regulators are still able to show basal expression of the σ^A -dependent P_{bmy} .

DegU binds at two sites in the bacillomycin D promoter region. The actions of DegU and DegQ are strongly interconnected. DegU stimulates the expression of *degQ*, but DegQ needs DegU in order to regulate *sacB* (29). While both proteins were found to be important for optimal *bmy* expression, which of the two proteins exerted a direct effect on P_{bmy} ? Inducing DegQ production in a *degU* mutant strain did not relieve bacillomycin D expression or production, as demonstrated by primer extension and MALDI-TOF MS (see Fig. S1 in the supplemental material). This means that the effect of DegU on *bmy* expression is epistatic to that of DegQ and that the latter needs the former in order to exert its role.

To examine whether the *bmy* promoter is a direct target of DegU, gel retardation EMSAs of a 450-bp DNA fragment harboring the region between bp -342 and $+108$ relative to the transcriptional start were performed using increasing concentrations of the response regulator. As seen in Fig. 4A, the *bmyD* promoter contains specific binding sites for DegU. The DNA fragment was slightly shifted with 0.2 and 0.4 μM unphosphorylated DegU, in a manner analogous to that for the DegU binding to the *comK* promoter (17). The mobility of the 450-bp DNA fragment changed dramatically upon incubation with higher protein concentrations (0.8 and 1.6 μM), indicating the presence of more than one DegU binding site at the promoter region of *bmy*. The observed shifts are DegU specific, since no DNA fragment migration was observed upon incubation of the DNA fragment with the same amounts of bovine serum albumin (Fig. 4A).

FIG. 3. DegU and ComA control the expression of bacillomycin D. (A and B) Strains AK9 and AK10, carrying the two *bmyD::lacZ* fusions with the longest upstream regions (see also Fig. 2A), and their *degU* (A) and *comA* (B) mutant derivatives were grown in Difco medium at 37°C, and optical densities (OD) (closed symbols) and β -galactosidase activities (open symbols) were determined during growth. Squares, AK9; diamonds, AK10; triangles, mutant derivatives of AK9 (AK32 in panel A and AK22 in panel B); circles, mutant derivatives of AK10 (AK33 in panel A and AK23 in panel B). (C) Effects of *degU* and *comA* mutations on the activity of the *bmy* operon promoter (P_{bmy}). The intensities of the obtained transcripts indicate the effects exerted by the respective mutations on the activity of the P_{bmy} promoter. 1, FZB42; 2, TF1 (*degU*); 3, CH23 (*comA*). +1 and the adjacent arrow indicate the transcriptional start.

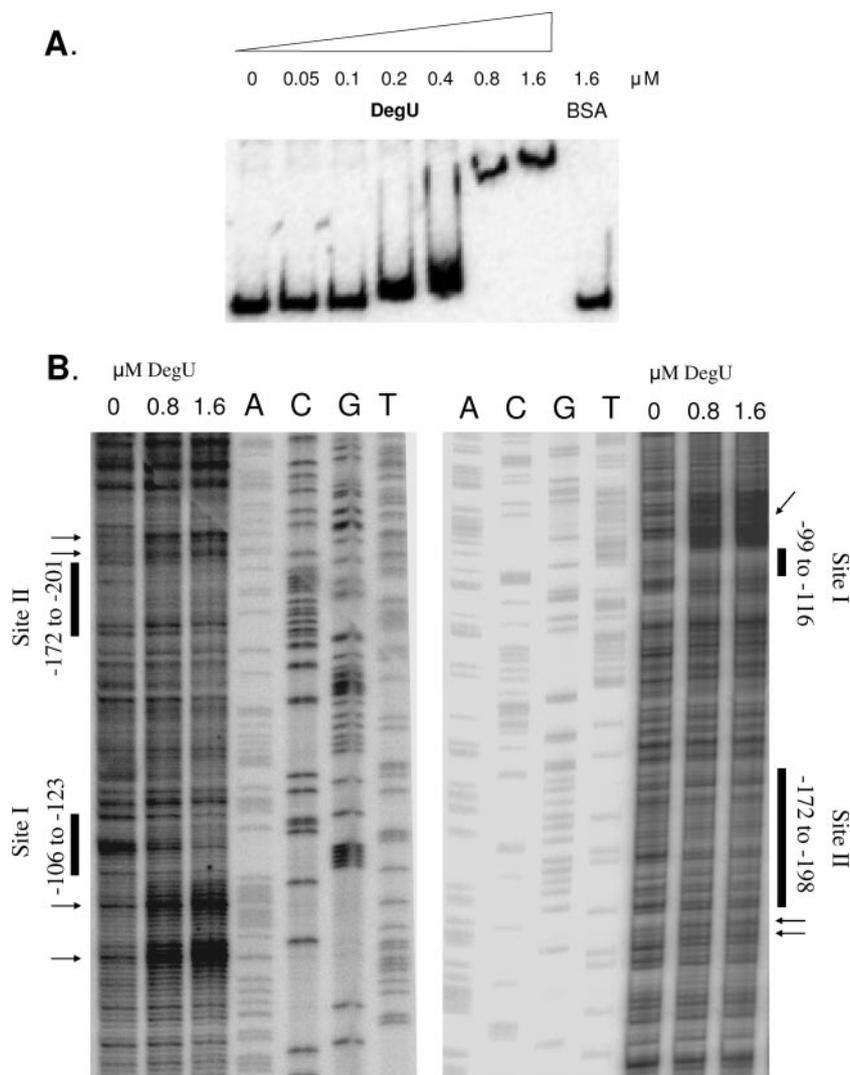


FIG. 4. DegU binds directly to the *bmy* promoter. (A) Gel retardation mobility shift assays of a ^{32}P -labeled *bmyD* promoter fragment using increasing concentrations of DegU, as indicated. The DNA fragment used harbors regions between bp -342 and $+108$ relative to the transcriptional start. (B) DNase I footprinting of DegU at the *bmy* promoter region. The DNase I digestion patterns were obtained using primer extension in order to be able to monitor both linear and supercoiled DNA; results were very similar with both types of DNA, and the results with supercoiled DNA are shown. The footprints of the coding and noncoding strands in the presence of increasing concentrations of DegU can be seen in the left and right panels, respectively. The protected and hypersensitive regions are marked with bars and arrows, respectively. The sequence reactions of the appropriate DNA strands were used as size markers.

Since DegU affects the expression of both the shorter and the longer versions of the *bmyD::lacZ* fusions (present in strains AK9 and AK10 [Fig. 3A]), we performed EMSA with two smaller DNA fragments. Fragment D1 encompassed the region between bp -342 and -126 relative to the transcriptional start, which is present only in AK9, while fragment D2 encompassed the DNA region between bp -125 and 108 bp, which is present in both AK9 and AK10. EMSA experiments with fragments D1 and D2 and increasing concentrations of the response regulator confirmed that unphosphorylated DegU directly binds to both DNA regions, with similar affinities (data not shown).

The exact locations of the DegU binding sites at the *bmy* promoter region were determined by DNase I footprinting. The footprinting patterns obtained with the coding and the

noncoding strands led to similar conclusions. In the presence of DegU, a region spanning from bp -123 to -106 on the coding strand was protected. The protected area was followed by an extended region of hypersensitive sites ranging from bp -103 to -85 . A second protected area with adjacent hypersensitive sites was identified between bp -201 and -172 on the coding strand. Consistently, the noncoding strand contained protected areas between bp -116 and -99 and bp -172 and -198 upon DegU addition. Hypersensitive sites could be observed in the region between bp -98 and -66 on the same strand (Fig. 4B).

Posttranscriptional control of bacillomycin D synthesis.

The assessment of the effects of the regulatory proteins DegU and ComA and of a *yczE* mutation on bacillomycin D synthesis was monitored by MALDI-TOF MS, which is an efficient and

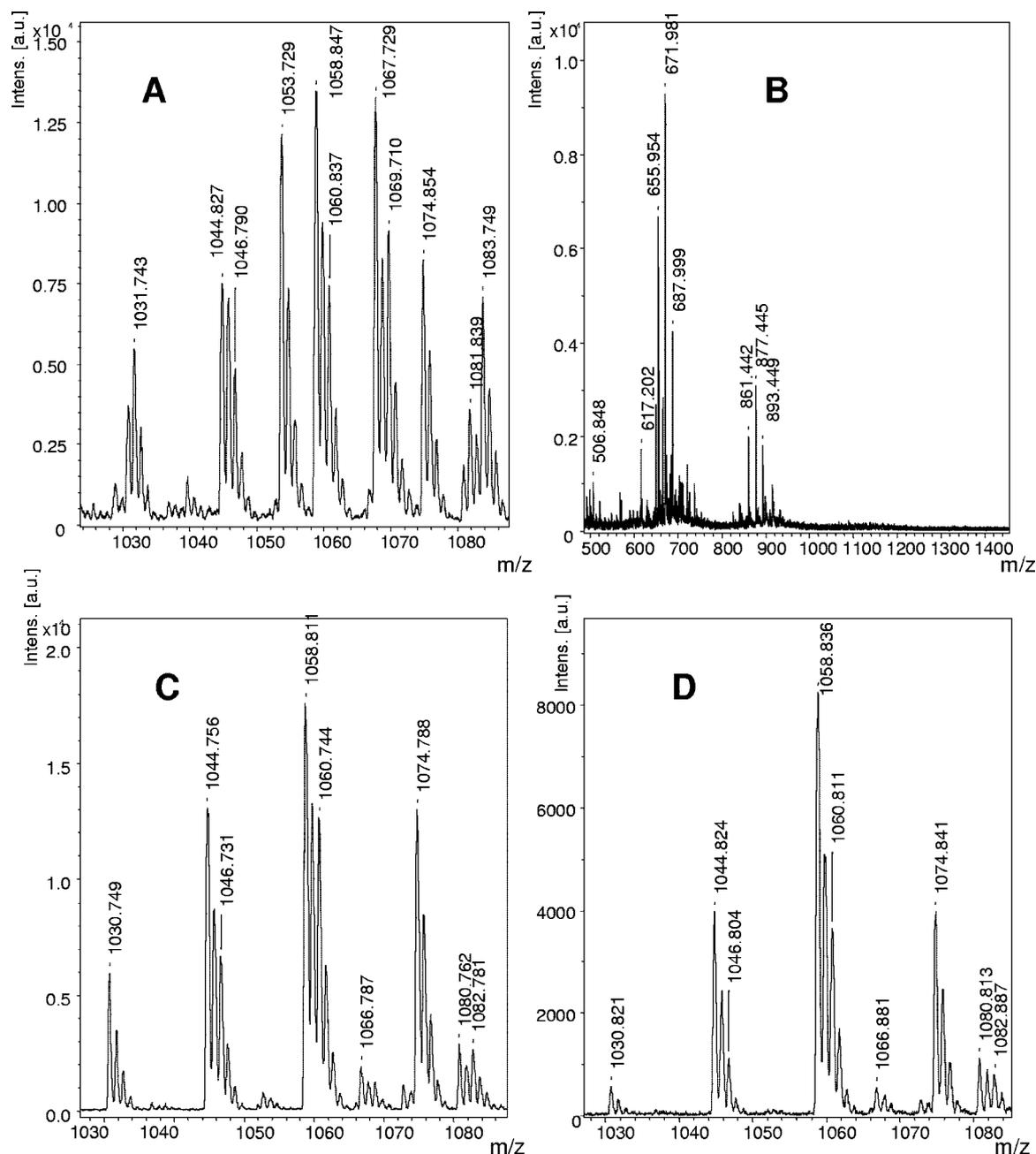


FIG. 5. MALDI-TOF MS of the lipopeptide products in culture filtrate extracts of the wild-type *B. amyloliquefaciens* FZB42 (A) and of the mutants CH3 (*sfp*) (B), TF1 (*degU*) (C), and CH4 (*yczE*) (D). C_{14-16} bacillomycin D-specific mass peaks at m/z 1031.7, 1053.7, 1067.7, 1069.7, 1081.7, and 1083.7 were present only in the supernatant of the wild type (A), while the mutant strains did not produce this antibiotic. The TF1 *degU* (C) and the CH4 *yczE* (D) mutant strains were still able to produce C_{13-15} surfactins, as indicated by the remaining mass peaks between m/z 1030 and 1090.

fast method to obtain qualitative and half-quantitative results for lipopeptides production in crude extracts in a minimum of time. As the reference, an MS of lipopeptide formation by the wild-type strain FZB42 is shown in Fig. 5A. Its main product is bacillomycin D, as judged from the intensities of the mass peaks at m/z 1031.7, 1053.7, 1067.7, 1069.7, 1081.7, and 1083.7, which can be attributed to sodium and potassium adducts of isoforms with fatty acid side chains of 14 to 16 carbon atoms. They are found together with those of surfactins in the mass

range of m/z 1030 to 1090. The surfactin peaks appear at m/z 1030.8, 1044.8, 1046.8, 1058.8, 1060.8, and 1074.8. They are of lower intensity than the bacillomycin D peaks and correspond to surfactin species with fatty acid side chains of 13 to 15 carbon atoms. The peaks found at m/z 1463.8, 1477.8, 1485.8, 1487.8, 1499.8, 1501.8, 1513.8, 1515.8, 1527.8, and 1529.8, indicating C_{15} to C_{17} fengycins, show the lowest intensities of the lipopeptide products. In the MALDI-TOF MS of the wild-type strain (see Fig. S2A in the supplemental material), their inten-

sities were approximately 10-fold lower than those of bacillomycin D. In the MS of the *comA* mutant CH23, the intensities of the bacillomycin D peaks were strongly reduced, showing a size similar to those of the fengycin peaks, which are slightly higher than those in the spectrum of the wild-type strain. It can be estimated that in CH23, bacillomycin D formation was lowered to about 10 to 20% of that in the wild-type corroborating our previous findings on the influence of ComA on *bmyD* expression. Apparently, deletion of *comA* in FZB42 severely impaired but did not silence transcription of the *bmy* operon. P_{bmy} retained its basal expression at the transcription level. In addition, in agreement with previous results (42), the *comA* mutant strain is also deficient in surfactin production (see Fig. S2B in the supplemental material).

Mutant CH3 bears a mutation in the 4'-phosphopantetheinyl transferase Sfp that results in a complete deficiency of lipopeptide production as deduced from the MS of its culture filtrate (Fig. 5B). Surprisingly, bacillomycin D completely disappeared in a culture filtrate of the DegU mutant strain TF1 (Fig. 5C), suggesting that the role of DegU in bacillomycin synthesis goes beyond the mere activation of the P_{bmy} promoter during transcription. To test whether this effect was associated with the export of this lipopeptide to the extracellular milieu, a sonicated cell extract of the *degU* strain was analyzed by MALDI-TOF MS. However, in this case bacillomycin D also could not be detected (data not shown), suggesting that DegU influences both transcription of the *bmy* operon and a posttranscriptional step prior to its export to the surrounding environment.

Surprisingly, the disruption of the *yczE* gene, which is located adjacent to *sfp*, also completely abolished the production of bacillomycin D, whereas surfactin and fengycin biosynthesis remained unimpaired (Fig. 5D). Previous results have shown that the fungicidal action exerted by bacillomycin D can be used to indicate production of bacillomycin D (20). While production of bacillomycin D by FZB42 was visible as a distinct zone of inhibition within a lawn of the baker's yeast *Saccharomyces cerevisiae*, no fungicide production was visible in the *degU* and *yczE* mutant strains TF1 and CH4, respectively (Fig. 6A). To test whether YczE exerts bacillomycin D synthesis on the transcriptional level, the *yczE::Em^r* mutation was introduced into the $P_{bmyD}::lacZ$ fusion strains AK9 and AK10. The β -galactosidase activity was not influenced by the *yczE* mutation (see Fig. S3A in the supplemental material), suggesting that YczE acts posttranscriptionally on bacillomycin D synthesis. Since YczE encodes an integral membrane protein of 215 amino acids consisting of five predicted transmembrane spanning helices (<http://www.cbs.dtu.dk/services/TMHMM/>), its function could be implicated in the export of bacillomycin D. To test this hypothesis, a sonicated cell extract of the *yczE* mutant strain was analyzed by MALDI-TOF MS. Bacillomycin D was not found inside the cells (data not shown), indicating that YczE is involved not in the antibiotic's export but rather in its synthesis, in a yet-unidentified manner. Finally, it can be ruled out that the posttranscriptional effects of DegU and YczE on bacillomycin D synthesis are mediated through the same pathway, since DegU did not activate the *yczE* promoter (see Fig. S3B in the supplemental material).

Posttranscriptional control exerted by YczE is absolutely essential for bacillomycin D synthesis. In order to examine

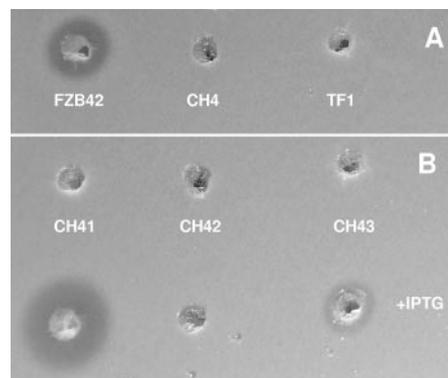


FIG. 6. Production of bacillomycin D by FZB42 derivatives as indicated by growth inhibition of the *Saccharomyces cerevisiae* indicator strain used in the agar diffusion assay. (A) FZB42, CH4 (*yczE*), and TF1 (*degU*). (B) Top, CH41 ($P_{bmyD}::P_{spac}$), CH42 ($P_{bmyD}::P_{spac} yczE$), and CH43 ($P_{bmyD}::P_{spac} degU$) without IPTG; bottom, CH41 ($P_{bmyD}::P_{spac}$), CH42 ($P_{bmyD}::P_{spac} yczE$), and CH43 ($P_{bmyD}::P_{spac} degU$) after induction by IPTG.

whether DegU and/or YczE is required for the posttranscriptional expression of bacillomycin D, we replaced the natural P_{bmyD} promoter with the IPTG-inducible P_{spac} promoter in FZB42 and the respective mutant strains. In the absence of IPTG, no bacillomycin D production was recorded. However, in the presence of IPTG, CH41, containing the IPTG-inducible promoter, expressed large amounts of bacillomycin D as indicated by its fungicidal action against baker's yeast. In contrast, IPTG did not turn on bacillomycin D production in strain CH42, bearing the same promoter but deficient in *yczE*. In strain CH43, harboring the *degU* mutation, production of bacillomycin D was strongly reduced but not completely silenced (Fig. 6B).

DISCUSSION

We have demonstrated here that the *bmy* operon, governing nonribosomal synthesis of the cyclic lipopeptide bacillomycin D, the main component of antifungal compounds produced by FZB42, is under direct transcriptional control of DegU, in contrast to the related cyclic lipopeptide surfactin, which is part of the ComA regulon (31, 36). Expression of *bmy* is dependent on a σ^A -dependent promoter, P_{bmy} , which is activated by interaction with DegU during early stationary phase. A similarly organized promoter has been reported to control the expression of iturin A in *B. subtilis* RB14 (44), though the reported transcriptional start, which is situated 1 bp downstream of our main transcriptional start, differs slightly from the one identified here. Although a direct interaction between DegU and the iturin promoter has not been investigated, it is very likely that expression of the giant gene clusters governing synthesis of iturin-like compounds in bacilli (12, 18, 44) are controlled by the same transcriptional regulator.

DegU binds to and activates P_{bmy} . DegU is a two-component system response regulator that controls many cellular processes, including exoprotease production, competence development, motility, and osmotic response, and triggers post-exponential-phase responses under growth-limiting conditions (9, 10, 22, 39). Here we have pinpointed a key function of

DegU in transcription regulation of a nonribosomally synthesized antibiotic. A series of in vivo and in vitro data indicated that DegU directly activates the expression of P_{bmy} . The results from the EMSA and the DNase I footprinting experiments coincided and indicated that DegU occupied two distinct DNA binding sites at the *bmy* promoter. The first site, site I, is located close to the transcriptional start, between bp -123 and -99, whereas the second one, site II, is situated further upstream, between bp -201 and -172. Binding of DegU to site II is essential for the optimal activation of the promoter (Fig. 3A). We propose that the binding of DegU to site I triggers a sharp DNA bend directly downstream of it (seen as hypersensitivity in the footprinting analysis), thus enabling the site II-bound DegU to activate the promoter. This is a rather common transcriptional activation mechanism (4).

The protection that DegU offers to the DNA at its two binding sites is relatively weak, but strong hypersensitive sites can be observed adjacent to the two DNA binding sites, implying that the binding of DegU to its sites rearranges the local DNA architecture, probably by inducing strong DNA bending, constraint, or even unwinding, which makes the DNA more accessible to DNase I attack. Similarly, DegU was proposed to activate the *comK* promoter by altering the shape of the approximately four DNA helices that separate the tandem ComK boxes and thereby facilitating the binding of ComK to them (17).

There are several reasons why the binding of DegU to the DNA only weakly protects the latter against DNase I attack. First, in both our experiments and the previous study (17), unphosphorylated DegU was used for the footprinting analysis. Although in many studies response regulators are used in their unphosphorylated state in order to demonstrate DNA binding, the use of the phosphorylated form often results in more distinct/extended regions being protected against DNase I cleavage (8, 36). Therefore, footprinting analysis was also performed with DegU after incubation with "cold" acetyl phosphate, and results very similar to those with the unphosphorylated DegU were obtained. Even though incubation of a response regulator with acetyl phosphate should result in its phosphorylation, no direct proof that transphosphorylation actually took place can be provided. Another reason for the weak protection patterns of DegU is the nature of its binding sites. A/T-rich DNA regions, such as the DNA binding sites of DegU, are more curved and therefore less accessible to DNase I, even when the DNA is naked without any protein bound to it. Thus, the A/T-rich DNA binding sites appear to be "protected," even in the absence of their binding partner.

Despite the fact that studies directly monitoring the binding of DegU to DNA promoter regions in vitro are limited (17, 32, 37), two possible motifs have been proposed as putative DegU recognition sites. In vitro data from the *aprE* and *comK* promoters indicated that DegU recognizes an A/T-rich motif (37), whereas in vivo studies with the *wapA* promoter and an alignment of DegU-regulated promoters indicated AGAA-N₁₁-TTCAG as the recognition site for DegU (10). Although none of these studies provides conclusive evidence and they contradict each other, degenerate forms of the latter motif could be identified in the DegU protected regions within sites I and II at the *bmy* promoter region, whereas the A/T-rich motifs proposed as putative DegU binding sites were part of the hyper-

sensitive sites that were generated at the *bmy* promoter region upon addition of DegU in the DNase I footprints. In any case, further experimental evidence, involving extensive site-directed mutagenesis, will be required to identify the consensus sequence recognized by DegU in P_{bmy} and/or other promoters.

It is generally assumed that DegU has two modes of action: phosphorylated DegU (DegU-P) directly activates degradative enzyme production and represses motility, whereas unphosphorylated DegU directly stimulates competence (9) through binding to the *comK* promoter (17). The assumption that only unphosphorylated DegU is required for competence has been based on the observation that either hyperphosphorylation of DegU or complete inactivation of the *degSU* operon decreases competence, whereas inactivation of *degS* alone leaves competence unaffected. Moreover, a DegU mutant with an impaired phosphorylation site has no effect on competence (9). However, there are alternative explanations of why neither the hyperactive form of DegU nor the complete absence of DegU allows competence, whereas the modest activity of the unphosphorylated DegU is sufficient to activate competence. DegU has opposing effects on different members of the DNA uptake gene cascade. On one hand, it coactivates the *comK* promoter (17), but on the other hand, it represses the *srf* operon (24) and therefore also inhibits the expression of *comS*. Reduced ComS levels result in an enhanced MecA/CipCP-mediated degradation of ComK (47). Thus, it may well be that the final action of DegU on *comK* expression is only positive, when the levels of DegU and/or its DNA binding affinity are relatively low (the unphosphorylated DegU form has probably a weaker binding affinity to its DNA targets). In contrast, the negative effect on *comS* expression prevails when the cellular amounts or the activity of DegU increase. This scenario is supported by recent microarray results (33), in which the DegU regulon was defined by comparing a *degS* mutant with its isogenic strain bearing a DegU-containing plasmid. *comK* was not part of the induced genes, whereas all the phosphorylated DegU-dependent genes were. This means that the phosphorylation state of DegU alone does not define the targets of DegU. In our case, DegU activates the expression of the *bmy* operon during stationary-phase growth. Therefore, it seems plausible that DegU-P is more suitable for optimal promoter binding and activation. However more direct evidence is required to verify this suggestion.

DegQ positively affects *bmy* expression via DegU. DegQ is a small pleiotropic regulatory protein, which consists of 46 amino acids and controls the expression of degradative enzymes, intracellular proteases, and several other secreted enzymes (1, 29). Recently it was also shown to enhance the production of the peptide antibiotics plipastatin and iturin A (45, 46). Here we demonstrate that DegQ transcriptionally activates the biosynthesis of a nonribosomally synthesized antibiotic and therefore is important for the high production of bacillomycin D in its host strain. However, the effects of DegQ are only indirect and are mediated via DegU for a number of reasons: (i) DegQ shares no homology to typical transcriptional regulators, i.e., DNA binding proteins; (ii) DegQ overexpression cannot complement the loss of DegU in terms of bacillomycin D synthesis; and (iii) DegQ affects *bmy* expression only when both DegU recognition sites are kept intact in the *bmyD* promoter region (Fig. 2D). Our results corroborate the

earlier observation that DegQ controls the expression of *sacB* (encoding a levansucrase) only in the presence of DegU (29). We suggest that DegQ modulates the activity of DegU, via an unknown mechanism. Interestingly, DegQ shows homology to a small region of the eukaryotic A kinase anchor proteins (11). Therefore, a possible role could be that it facilitates the transphosphorylation to DegU.

ComA probably affects *bmy* expression via DegQ/DegU. While DegU was shown to directly bind to the *bmy* promoter region, it is likely that ComA controls *bmy* expression indirectly via DegQ, which serves as an auxiliary factor to DegU. The absence of putative ComA binding boxes within the *bmyD* upstream region supports this assumption. However, the two ComA boxes found in the *degQ* gene in *B. subtilis* are conserved in FZB42, and therefore ComA likely promotes the expression of *degQ* in this organism, as has been shown in *B. subtilis* (29). Unfortunately, it was impossible to verify the proposed indirect role of ComA, as we were unable to obtain a *comA*-deficient mutant of FZB42 in which *degQ* is expressed from an IPTG-inducible promoter.

DegU and YczE posttranscriptionally control bacillomycin D production. Deletion of both *degU* and *yczE* specifically abolished the production of bacillomycin D without influencing the production of other peptide antibiotics produced by FZB42. While DegU has an additional effect on P_{bmy} activity, YczE exerts its effect only in a posttranscriptional manner. Neither is involved in the export of the lipopeptide into the external milieu, and they exert their effects through independent pathways. This conclusion is supported by the results obtained with strains in which the IPTG-inducible P_{spac} promoter was inserted in order to replace the natural P_{bmy} promoter. Efficient synthesis of bacillomycin D occurred only in the wild-type background, while in the presence of the *degU* mutation, the P_{spac} -dependent synthesis was strongly reduced. In the presence of the *yczE* mutation, the bacillomycin D synthesis was completely abolished, suggesting that *yczE* is essential for an unknown posttranscriptional event during synthesis.

A recent report (43) describes the existence of a membrane-bound, organelle-like protein complex responsible for synthesis of the polyketide bacillaene in *B. subtilis*, a process resembling nonribosomal synthesis of lipopeptides. It can be speculated that the giant nonribosomal peptide synthetase involved in bacillomycin D synthesis in FZB42 assumes a similar superstructure, and the integral membrane protein YczE is a candidate for anchoring bacillomycin D synthetase at the membrane.

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