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Two-Component Response Regulator DegU Controls the Expression of Bacilysin in Plant-Growth-Promoting Bacterium *Bacillus amyloliquefaciens* FZB42

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Key Words
Bacillus amyloliquefaciens FZB42 · Bacilysin · DegU · ScoC

Abstract
The plant-growth-promoting-rhizobacteria *Bacillus amyloliquefaciens* FZB42 possess an enormous potential to synthesize a wide range of antimicrobial, antiviral and nematocidal compounds. One of them, the dipeptide antibiotic bacilysin, is synthesized by FZB42 during exponential growth. Here, we have demonstrated that bacilysin is positively regulated by the two-component response regulator DegU at the transcriptional level. In addition, ScoC (Hpr), a transition state regulator, negatively controlled expression of the *bacA* gene, which is the first gene within the bacilysin operon. Both DegU and ScoC were bound directly at the *bac*A promoter region. Furthermore, a monocistronic gene located in close vicinity of the *bac* operon and essential for bacilysin production, *ywfH,* was also regulated by DegU. Transcription of the *bac* operon and of the *ywfH* gene in *B. amyloliquefaciens* FZB42 was positively controlled by the DegU global regulator protein. The role of interactions within a ternary complex formed by the antagonistically acting regulators DegU and ScoC as well as the *bacA* promoter sequence remains to be elucidated.

Introduction

The *Bacillus amyloliquefaciens* subsp. *plantarum* strain FZB42 is a plant-growth-promoting, root-colonizing and pathogen-suppressing representative of plant-associated bacilli, and is closely related to *Bacillus subtilis* [Borriss et al., 2011]. Its genome contains numerous gene clusters involved in nonribosomal synthesis of lipopeptide and polyketide antibiotics [Chen et al., 2007]. While nonribosomal synthesis of lipopeptides and polyketides is dependent on phosphopantheine transferase, Sfp, nonribosomal synthesis of the antimicrobial dipeptide bacilysin is not dependent on Sfp [Chen et al., 2009a]. Recently, a ribosomal synthesized peptide with antibacterial activity and also contributing to the successful competing of *B. amyloliquefaciens* FZB42 in the rhizosphere was detected in FZB42 [Kalyon et al., 2011; Scholz et al., 2011].

Nonribosomal synthesized bacilysin (L-alanyl-(2,3-epoxycyclohexanone-4)-L-alanine), a dipeptide consisting of non-proteinogenic L-anticapsin and N-terminal L-alanine, was identified, firstly, in *B. subtilis*. [Rogers et al., 1965]. Despite its simple structure, it has an impressive antimicrobial activity against a wide range of bacteria and some fungi [Walker and Abraham, 1970]. The mode of antimicrobial activity is mainly due to the inhibition

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of glucosamine synthesis, leading to defects in the microbial cell wall [Kenig et al., 1976; Walton and Rickes, 1962]. It has been well established that prephenate, an intermediate of the aromatic amino acid biosynthetic pathway, is the primary metabolic precursor of the anticapsin moiety [Mahlstedt and Walsh, 2010; Roscoe and Abraham, 1966]. Anticapsin is released from bacilysin upon transport into the cell, making the essence of bacilysin antimicrobial activity [Perry and Abraham, 1979]. Bacilysin is also expressed by plant-associated *B. amyloliquefaciens* FZB42 [Chen et al., 2009a]. We demonstrated that bacilysin, together with the polyketide difficidin, acts antagonistically against the phytopathogenic bacterium *Erwinia amylovora*, the causative agent of fire blight disease in orchard trees [Chen et al., 2009b].

Bacilysin biosynthesis is encompassed by the *bacABCDE* operon [Steinborn et al., 2005]. In addition, a monocistronic gene, *ywfH*, is also essential for the complete cycle of bacilysin formation [Inaoka et al., 2003]. Gene products of *bacA* and *bacB* are involved in converting prephenate into dihydro-4-hydroxyphenylpyruvate (H_2HPP_4) and dihydro-5-hydroxyphenylpyruvate (H_2HPP_5), respectively. The *ywfH* gene product is involved in converting H_2HPP_5 to H_4HPP_6, making the precursor for anticapsin; the function of *bacC* is unclear. The *bacD* and *bacE* gene products direct amino acid ligation and host bacterium self-protection, respectively [Mahlstedt and Walsh, 2010]. The *bacABCDE* gene cluster in FZB42 is collinear to *B. subtilis* 168, and its homology ranges between 84 and 93% [Chen et al., 2009a].

Regulation of bacilysin expression in *B. subtilis* has been the subject of several studies. Bacilysin synthesis is controlled by a dual regulation system composed of the guanine nucleotides ppGpp and GTP sensed by CodY mediating repression in *B. subtilis* [Inaoka et al., 2003]. In addition, the bacilysin operon is under the control of the competence-regulating protein ComA, and negatively regulated by the transition state regulator AbrB [Yazgan et al., 2001]. Three Phr peptides, PhrC, PhrF and PhrK, are required for full-level expression of ComA-dependent *bac* operon expression [Koroglu et al., 2011]. Recently, it was reported that, in *B. subtilis*, transition state regulator ScoC (Hpr) represses bacilysin transcription by direct promoter binding [Inaoka et al., 2009].

In this study, we found that regulation of the *bac* operon in environmental *B. amyloliquefaciens* FZB42 resembled that which was previously described in *B. subtilis* 168. However, in addition to the transcriptional regulators known before, we found that bacilysin transcription was under direct positive control of the two-component global regulator DegU, while the transition state regulator ScoC negatively affected bacilysin production in FZB42.

Results

Identification of Transcriptional Start Sites of the bac *Operon and of the* ywfH *Gene*

Transcriptional start points of *bacA,* the first gene of the *bac* operon, and of the monocistronic *ywfH* were determined using primer extension (fig. 1a). For the *bacA* gene, an adenine, located 23 bp upstream of translation start (ATG) was identified as +1. A highly conserved –10 region (TAATAT) and a conserved –35 region (TTGACA) specific for sigma factor A (σ^A) could be identified in appropriate distances to +1. Additionally, we detected a TATG motive located around –16 and a possible UP element (–47 to –54) that is commonly composed of a poly-A (–51 to –54) and a poly-T (–47 to –50) sequence [Voskuil and Chambliss, 1998]. The ribosomal binding site was less well conserved in the *bacA* upstream region (fig. 1c, top).

According to the strong signal corresponding to the transcriptional start point, transcription of the *ywfH* gene seemed to be more pronounced than *bacA* (fig. 1b). We identified an adenine located 21 bp upstream of the translation start TTG as start of transcription of *ywfH*. In contrast to *bacA*, P_{ywfH} did not possess consensus –35 and –10 boxes. However, the ribosome-binding site AGGAGAT was well conserved (fig. 1c, bottom).

Mapping of bacA *Promoter Activity*

In order to monitor the transcriptional regulation of bacilysin synthesis, four reporter fusions of the *bacA* promoter region to *lacZ* were generated. A fifth fusion between the full-length *ywfH* promoter with *lacZ* was also generated (fig. 2a). A series of nested DNA fragments with a common downstream end (bp +221 relative to the transcriptional start) and variable upstream ends (bp –671, bp –114, bp –82, bp –36 relative to the transcriptional start) were fused to a promoterless *lacZ* gene in pAK9 [Koumoutsi et al., 2007]. The transcriptional fusions were introduced as single copies into the chromosome of AA1, and their expression was measured throughout the growth cycle (see Experimental Procedures). All variants displayed similar growth kinetics in GA medium (fig. 2b). β-Galactosidase expression in strain AA2, bearing the 892-bp full-length promoter region, including the 3′ part of the coding region for the *ywfA* gene,

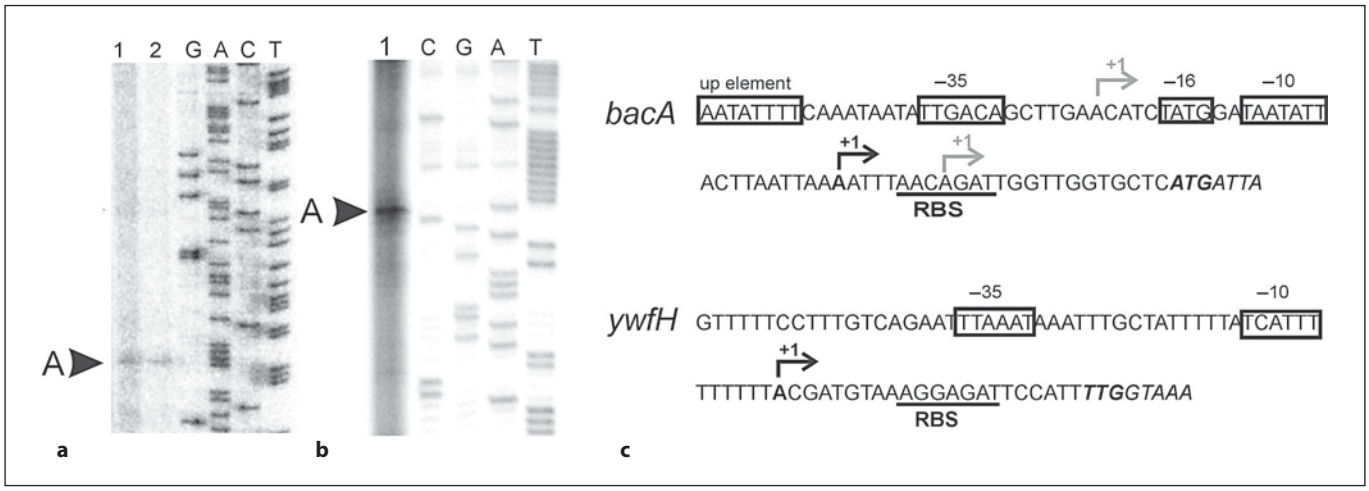
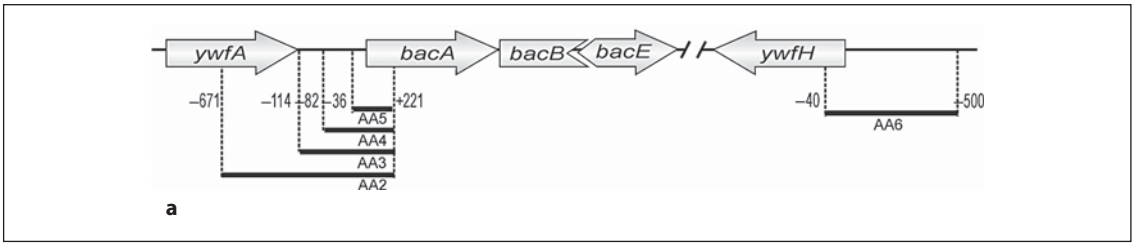


Fig. 1. Mapping of the transcriptional start sites by primer extension analysis. **a** Primer extension of the *bac* operon was performed using 5′-^{32}P end-labeled primer PeRW3 (table 2). The four lanes labeled as GACT result from dideoxynucleotide sequencing reactions. The black arrowhead at the left side indicates the position of the main transcription start. Minor transcriptional start points are indicated by the two grey arrowheads. **b** Primer extension of the *ywfH* gene was performed using 5′-^{32}P end-labeled primer YwfH PE2 (table 2). The black arrowhead indicates the transcriptional start site. **c** The corresponding DNA promoter sequences from *bacA* (top) and *ywfH* are presented at the bottom. The position of the transcriptional start sites (boldface, +1), putative ribosome-binding sites (underlined); –35 and –10 hexamers and –16 tetramer (boxes), and the coding sequences (italics) are indicated.



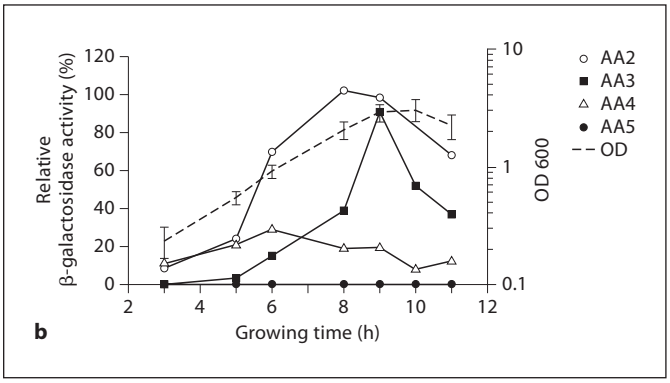


Fig. 2. Mapping of the functional *bacA* promoter region by 5′ deletions. **a** Schematic representation of the *bac* gene cluster with flanking regions, including the *ywfH* gene which is in opposite orientation. The different *bacA::lacZ* fusions AA2 to AA5 are denoted with their nucleotide position relative to the transcriptional start. Fusion AA6 corresponds to the full-length promoter of *ywfH*. **b** Expression of the series of *bacA::lacZ* fusions with different 5′ end termini in FZB42. The cells were grown in GA medium for 12 h.

increased steadily during the logarithmic growth phase and reached its maximum after a total of 8 h upon entry into transition phase. Removal of the *ywfA* coding region (AA3) lowered gene expression during the exponential phase, but its activity maximum after 9 h of growth was only slightly reduced (92% compared to AA2). Promoter activity during the stationary phase was more reduced in strain AA3. After shortening of the upstream promoter region to –82, reporter activity was only 20% compared to AA2. Furthermore, in this mutant the promoter activ-

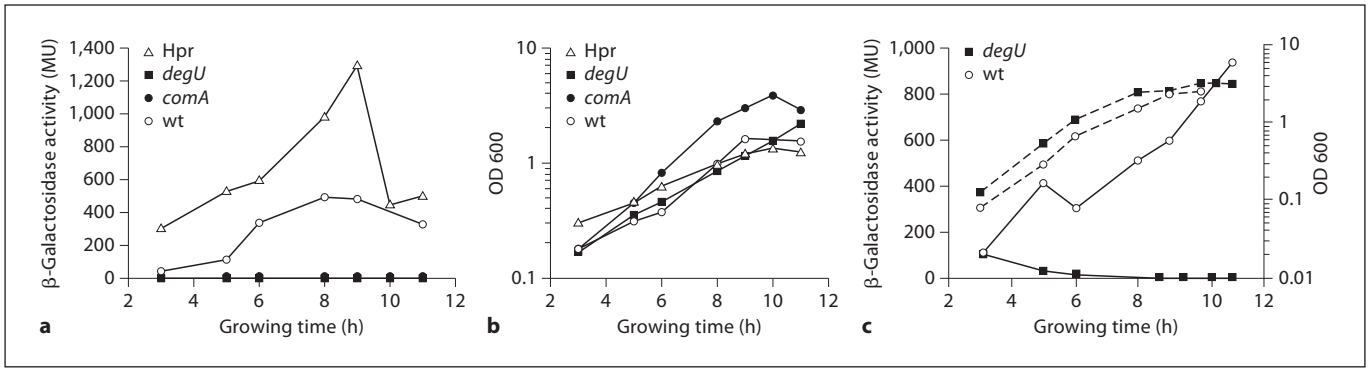


Fig. 3. DegU, ScoC (Hpr), and ComA control *bacA* and *ywfH* promoter activities. Strains AA2 (wt), AM11 (Δ*comA*), AM12 (Δ*degU*) and AM13 (Δ*scoC*) carrying the *bacA::lacZ* fusion, and AA6 (wt) and AM14 (Δ*degU*) carrying the *ywfH::lacZ* fusion were grown in GA medium and optical densities and β-galactosidase activities were determined during growth. **a** BacA expression is derepressed in the *scoC* mutant while *comA* and *degU* mutant strains did abolish *bacA* promoter activity. **b** Growth of *bacA::lacZ* fusion strains. **c** Expression of *ywfH* gene is steadily increasing during exponential and transitional growth phase. The *ywfH* promoter activity is completely repressed in the *degU* mutant background. Growth of AA6 and AM14 in GA medium is indicated by a dotted line. The growth kinetics of the mutant strains displayed in **a** and **b** resembled that of AA6 and AM14, respectively.

ity dropped during the stationary phase. These results indicated that the main binding sites of the positive regulator(s) might be located within –114 and +221. However, further effectors might also bind upstream of –114. Strain AA5 (–36), where the upstream promoter region was completely removed, exhibited no promoter activity.

The Global Transcriptional Regulators DegU, ComA and ScoC Control Promoter Activity of bacA *in FZB42*

Detailed analysis of the *bac* promoter activity was carried out with derivatives of strain AA2, bearing the full-length *bacA* promoter region (–671 to +221) fused with the reporter gene *lacZ*. The *bac* promoter-driven β-galactosidase activity was measured in wild-type and in mutant strains deficient in expression of the global regulators ComA (AM11), DegU (AM12) and ScoC (Hpr; AM13). While the wild-type background strain (AA2) expressed about 500 MU of β-galactosidase after 9 h at the transition phase, the *scoC* gene-deficient strain AM13 exhibited an uncoupled synthesis of β-galactosidase reaching 1,400 MU, thereby corroborating earlier results obtained with *B. subtilis* [Inaoka et al., 2009]. BacA promoter-dependent reporter gene activity was completely abolished in *comA* and *degU* mutants (fig. 3a, b).

The ywfH *Promoter Is Controlled by DegU*

In addition, we demonstrated that the *ywfH* promoter was also depending on the global regulator DegU. The 540-bp region upstream of *ywfH* was fused to *lacZ*, and β-galactosidase activities were measured in both wild-type (strain AA6) and in the *degU* mutant (strain AA14) during the growth cycle. The *ywfH* promoter was active during exponential growth, but in contrast to the *bac* promoter, no decrease after entering the stationary phase was detected. The *degU*-deletion mutant exhibited no promoter activity indicating the positive effect of DegU on *ywfH* gene expression (fig. 3c).

DegU Binds Directly at the bacA *and* ywfH *Promoter Region*

According to our results obtained by using *lacZ* promoter fusions, ComA and DegU were involved in transcriptional regulation of *bacA* gene expression. To examine whether the *bacA* promoter is a direct target of DegU, gel retardation assays, EMSAs, were performed using increasing concentrations of the unphosphorylated and phosphorylated response regulator DegU together with a *bacA*-fragment spanning between –171 and +93 and containing all putative *degU*-sites that were predicted in silico. Both forms of DegU shifted the *bacA* promoter in a concentration-dependent manner. Remarkably, unphosphorylated DegU bound more strongly to the *bacA* promoter than DegU~P (fig. 4a, b). This finding is in line with the results previously obtained with the *bmyD* promoter of FZB42, which was found to be more affected by unphosphorylated than by phosphorylated DegU [Koumoutsi et al., 2007].

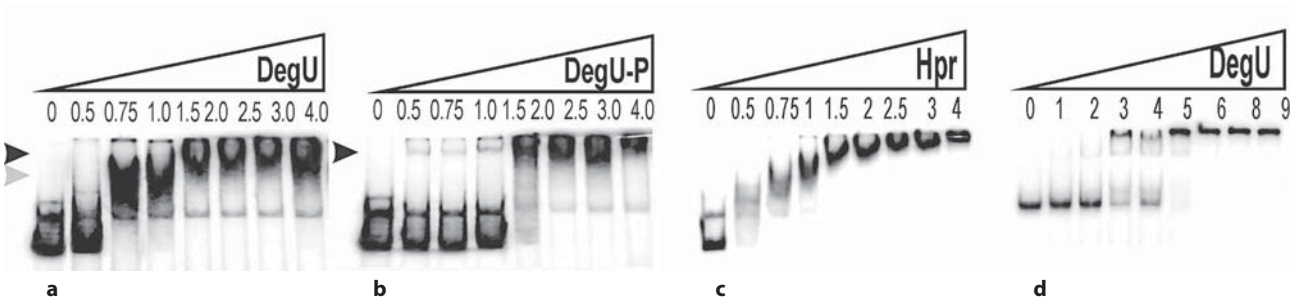


Fig. 4. Gel-retardation-mobility-shift assays of a ^{32}P-labeled *bacA* 170-bp promoter fragment (**a–c**) and a ^{32}P-labeled *ywrH* 160-bp promoter fragment (**d**) using increasing concentrations of DegU (**a**, **b**, **d**) and ScoC (**c**). Unphosphorylated (**a**) and phosphorylated DegU (**b**), and ScoC and bind directly to the *bacA* promoter. Adding of unphosphorylated DegU did also shift the *ywrH* 160-bp promoter fragment (**d**). Primers used for this study are listed in table 2. Protein concentrations (μM) are indicated at the top.

Since unphosphorylated DegU bound more strongly to the *bac* promoter, we used unphosphorylated DegU for DNase I footprinting. The experiments were carried out with a radio-labeled 264-bp fragment of *bacA* and a 170-bp fragment of *ywfH*. The fragments were incubated with rising concentrations of DegU, then treated with DNase I, and separated on sequencing gels (Experimental Procedure).

On the coding strand of the *bacA* promoter region, three regions covering –125 to –98, –82 to –74 and –57 to –5, respectively, were protected. These regions were separated by hypersensitive sites indicated by black arrowheads. On the template strand, only one protected region (–63 to –35) flanked by hypersensitive sites was found (fig. 5a). Interestingly, many less sensitive purines were present within hypersensitive regions, possibly indicating some structural changes of the DNA caused by DegU binding and resulting in inaccessibility of these bases for DNase I digestion.

Moreover, EMSA experiments revealed that DegU bound also to the *ywfH* promoter. A DNA fragment spanning the 160 bp between –119 and +39 of the *ywfH* promoter region was incubated with rising concentrations of unphosphorylated DegU. The *ywfH* promoter fragment was shifted at 2–3 μM DegU (fig. 4d).

Within the *ywfH* region, three protected regions were also identified by DNase foot printing: two were located within the promoter (–56 to –44, –37 to –14), and one was located within the transcribed region (–4 to +18). Only three hypersensitive bases were found here: one was located directly at the translation start (TTG), a second one was found downstream of site I, and a third within site II. This region (around –35) appeared bended in the absence of any protein because all bases showed an identical pattern in sequencing reaction (fig. 5c, gray line). Therefore, the position of the hypersensitive sites could not be determined exactly.

Similar to the *bmyD* [Koumoutsi et al., 2007] and the *pgsB* promoters [Ohsawa et al., 2009], DegU-mediated protection against DNase I digestion in *bacA* and *ywfH* regions was weak and difficult to detect (fig. 5a, c).

Hpr Binds to the bac *Promoter*

We used a purified His_6-tagged ScoC and a 693-bp fragment of the *bacA* region, spanning –671 and +22, to perform gel retardation assays. ScoC exhibited a higher affinity to the *bacA* promoter than DegU. Shifting of the DNA fragment started at relative low protein concentrations (0.5 μM), but mobility of the 693-bp fragment was gradually lowered to protein concentrations of 2.5 μM, suggesting that more than one ScoC protein interacted with the promoter (fig. 4c).

We also determined by DNase footprinting the ScoC-binding sites within a 173-bp fragment of *bacA*. The protection of ScoC was more pronounced than in DegU; 1 μM of ScoC was sufficient to protect against DNase I digestion, confirming the high affinity of this protein to *bacA* that we also observed in EMSA. We identified a shorter protected region within the coding region (+32 to +49) and a second region within the promoter region (+9 to –64). The latter was interrupted by a less protected region between –28 and –34. Between both ScoC-binding sites, a hypersensitive region was located (fig. 5b).

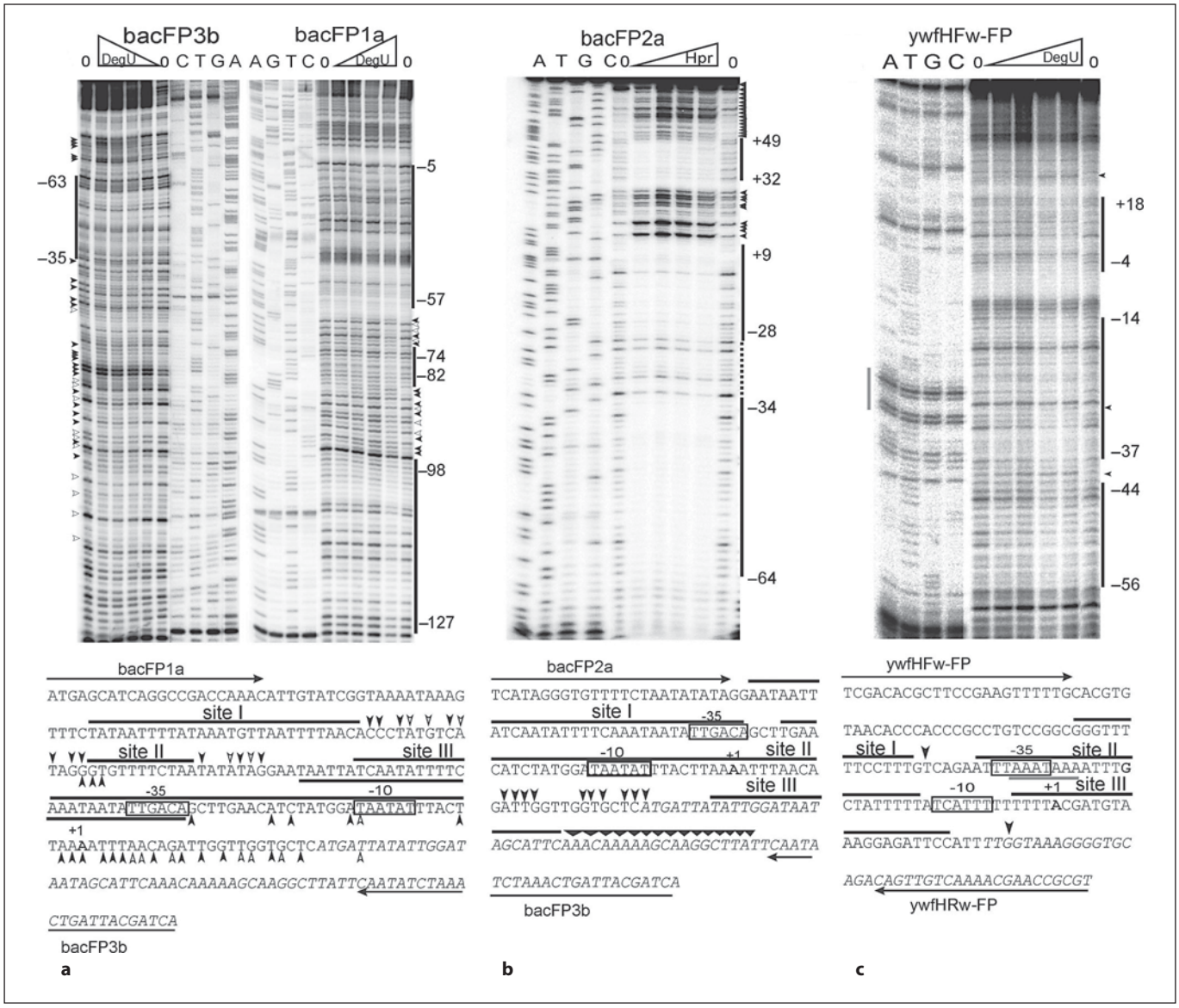


Fig. 5. Mapping of the DegU-binding site within the promoters of *bac* and *ywfH*. **a** The reverse and forward strands of the *bac* region were incubated with rising concentrations of DegU (1.0, 2.0, 5.0, 10.0 μM) and treated with DNase I. **b** DNase I footprinting of the forward strand of the *bac* region bound to ScoC (1.0, 2.0, 4.0, 8.0 μM). **c** DNase I footprinting of the forward strand of the *ywfH* region bound to DegU (concentrations of the protein were similar to **a**) The corresponding fragments' sequences are indicated below the DNase footprints. All symbols are identical for the images and sequences, as well. Protected sites are marked by black continuous lines, whereas the dotted line indicates a small region within bacA that exhibited a slightly reduced ScoC-mediated protection. Black arrowheads indicate hypersensitive sites, and white arrowheads mark single bases protected to DNaseI-digestion in presence of the protein. Primer positions are shown as horizontal arrows at the sequence.

Synthesis of Bacilysin and Difficidin in Regulatory Mutants of FZB42

Mutant strains deficient in the alternative sigma factors SigB, SigD, SigX, SigM, and SigV; the transcriptional regulators DegU, ComA and ScoC (Hpr); the phosphopantetheinyl transferase Sfp; and BacA (table 1) were constructed by using cassette mutagenesis as described previously [Koumoutsi et al., 2004], taking advantage of the natural competence for DNA uptake by FZB42. We used bioautography with *Bacillus megaterium* as an indicator

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference/origin
B. amyloliquefaciens		
FZB42	Wild type	Borriss et al., 2011
AK13	FZB42 *amyE*::P_0-*lacZ* (*Cm*^{r})	Koumoutsi, 2006
AA1	AK13 Δ *amyE*::P_{bac}0-*lacZ* (*Spec*^{r})	this study
AA2	AK13 Δ *amyE*::$P_{bac892bp}$-*lacZ* (*Cm*^{r})	this study
AA3	AK13 Δ *amyE*::$P_{bacbp335bp}$-*lacZ* (*Cm*^{r})	this study
AA4	AK13 Δ *amyE*::$P_{bac305bp}$-*lacZ* (*Cm*^{r})	this study
AA5	AK13 Δ *amyE*::$P_{bac257bp}$-*lacZ* (*Cm*^{r})	this study
AA6	AK13 Δ *amyE*::$P_{ywfH540bp}$-*lacZ* (*Cm*^{r})	this study
AM10	FZB42 Δ*hpr*::Spec^r Δ *amyE*::$P_{bac892bp}$-*lacZ* (*Cm*^{r})	this study
AM11	AA2 Δ*comA* ::Em^r Δ *amyE*::$P_{bac892bp}$-*lacZ* (*Cm*^{r})	this study
AM12	AA2 Δ*degU* ::Em^r Δ *amyE*::$P_{bac892bp}$-*lacZ* (*Cm*^{r})	this study
AM13	AA2 Δ*scoC* ::Em^r Δ *amyE*::$P_{bac892bp}$-*lacZ* (*Cm*^{r})	this study
AM14	AA6 Δ*degU* ::Em^r Δ *amyE*::$P_{ywfH540bp}$-*lacZ* (*Cm*^{r})	this study
TF1	AA6 Δ*degU* ::Em^r	Koumoutsi et al., 2007
RS06	FZB42 Δ*bacA,* Δ *sfp*::Em^r	Chen et al., 2009a
CH03	FZB42 Δ*sfp*::Em^r	Chen et al., 2009a
CH23	FZB42 Δ*comA*::Em^r	Koumoutsi et al., 2007
AK48	FZB42 *sigX*::*Km*^{r}	Koumoutsi, 2006
AK57	FZB42 *sigB*::*Em*^{r} *rapX*::*Cm*^{r}	
CH30	FZB42 *sigV*::*Em*^{r}	Chen, 2010
DH5α	*supE44 _lacU169 (_80 lacZ_M15) hsdR17 recA1 gyrA96 thi-1 relA1*	laboratory stock
BL21	F$^-$ *ompT hsd*S_B(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
AK38	*E. coli* DH5α pREP4 pAK54	Koumoutsi et al., 2007
Plasmids		
pGEM-T/ Promega	cloning vector, Ap^r	Promega
pET15b	expression vector, Ap^r	Novagen
pAK9	*B. amyloliquefaciens* FZB42 integration vector *amyE::lacZ*, Ap^r Cm^r, pDG268 derivative	Koumoutsi et al., 2007
pAFN1	pAK9 carrying a fragment of *bacA* from –671 to +221 bp	this study
pAFN2	pAK9 carrying a fragment of *bacA* from –671 to +221 bp	this study
pAFN3	pAK9 carrying a fragment of *bacA* from –114 to +221 bp	this study
pAFN4	pAK9 carrying a fragment of *bacA* from –82 to +221 bp	this study
pAFN5	pAK9 carrying a fragment of *bacA* from –36 to +221 bp	this study
pAYN1	pAK9 carrying a fragment of *ywfH* from –119 to +39 bp	this study
pAM07	pGEM-T carrying *abrB*::*Km*^{r}	this study
pAM10	pGEM-T carrying *scoC*::*Spec*^{r}	this study
pHPR	pET15b carrying *scoC*::Amp^r	this study

strain [Chen et al., 2006] to ascertain the amounts of bacilysin and difficidin produced by the different mutant strains in comparison to wild-type *B. amyloliquefaciens* FZB42 (fig. 6). Difficidin and bacilysin are known to inhibit the growth of *B. megaterium* [Chen et al., 2009b]. The *sfp* deletion mutant CH03, devoid in nonribosomal synthesis of lipopeptides and polyketides, was used for monitoring the bacilysin effect as a positive control. Strain RS06 (Δ*bacA* Δ*sfp*) unable to produce bacilysin and difficidin was used as negative control.

Deletion in the genes encoding the transcriptional regulators ComA and DegU yielded the strongest effects on bacilysin production. Thereby, deletion of *comA* solely inhibited bacilysin synthesis, whereas deletion of *degU* resulted in a negative effect on bacilysin and difficidin production. Knockout mutation of the global regulator

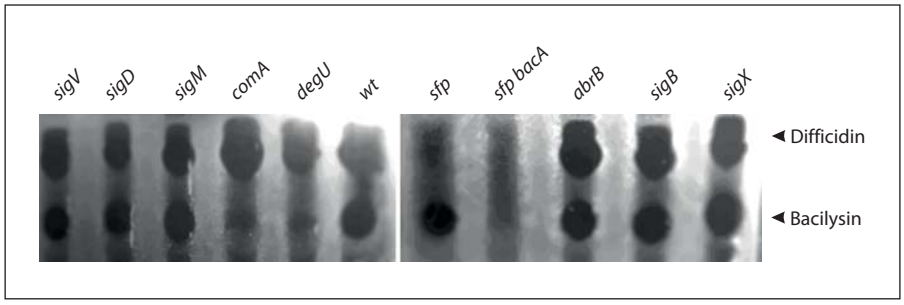


Fig. 6. Bioautography of FZB42 wild-type and mutant strains affected in synthesis of alternative sigma factors *(sigV, sigD, sigM, sigB)*, global regulators *(comA, degU, abrB)*, phosphopantheinyl transferase Sfp *(sfp)* and BacA *(bacA)*. Haloes indicate growth inhibition due to bacilysin and difficidin. *B. megaterium* was used as indicator strain.

AbrB did not affect either bacilysin or difficidin synthesis. Deletions of *sigB* and *sigX* were without effect on bacilysin and difficidin production, while deletions of *sigV*, *sigD* and *sigM* led to a slightly reduced production of difficidin, but did not affect bacilysin production.

Discussion

Recent experiments performed with *B. subtilis* have shown that ScoC inhibits expression of the *bac* operon at the transcriptional level [Inaoka et al., 2009]. We have demonstrated here that the global regulators DegU and ScoC act together in regulating bacilysin in *B. amyloliquefaciens* FZB42. DegU acts as a positive transcriptional regulator, while ScoC negatively controls transcription of the *bacA* promoter of *B. amyloliquefaciens* and thus expression of bacilysin in *B. amyloliquefaciens* FZB42. The regulatory network which directs bacilysin expression resembles that of bacillomycin D [Koumoutsi et al., 2007]. Therefore, DegU might be an important transcriptional activator of enzymes involved in nonribosomal synthesis of antimicrobial secondary metabolites in endospore-forming Gram-positive soil bacteria. This idea is additionally supported by results obtained with a *degU*-negative mutant strain which was found to be impaired in bacilysin and difficidin synthesis, too (fig. 6). Difficidin is an antibacterial polyketide which is nonribosomally synthesized in FZB42 [Chen et al., 2006]. As recently reported for *B. subtilis* [Koroglu et al., 2011], ComA, another global transcriptional regulator governing nonribosomal synthesis of cyclic lipopeptide surfactin [Nakano et al., 1991], is also necessary for expression of bacilysin in FZB42 (fig. 1, 6). We assume that ComA acts mainly indirectly through activation of DegQ, which in turn influences DegU by improving the phosphotransfer from DegS to DegU as proposed earlier for the regulation of *bmyD* in FZB42 [Koumoutsi et al., 2007].

Expression of the *bac* operon is dependent on a σ^A-dependent promoter, which is activated by interaction with DegU at the final stage of vegetative growth (fig. 1). According to our results obtained with mutants deficient in alternative sigma factors, it is unlikely that minor sigma factors are necessary for expression of the *bac* operon, although bacilysin production was found slightly reduced in the *sigV* and *sigD* mutant strains (fig. 6). The σ^A promoter for the *bac* operon of FZB42 was found to contain a well-conserved –35 and –10 region. In addition, it included a –16 sequence and a possible AT-rich 'UP element'. It is known that *B. subtilis* promoters containing a –16 region exhibit typical –35 and –10 regions and conserved T and A tracts at –40 to –54 that are similar to the UP element of rRNA-promoters in *Escherichia coli* [Voskuil and Chambliss, 1998]. Therefore, the σ^A-dependent *bac* promoter belongs to this promoter type.

Our EMSA studies revealed that unphosphorylated DegU exhibited higher affinities to the *bac* promoter than its phosphorylated forms. Since DegU becomes phosphorylated during the stationary phase of growth, this is in line with our finding that *bac* operon expression becomes decreased after the exponential growth phase.

Binding of DegU to the *bac* operon promoter occurred mainly at the coding strand. Three sites are involved. The largest site (III) overlapped the promoter at –10 and –35, and the possible UP element at –40. Another site (I) was located further upstream between –98 and –127. Between these two DegU-binding sites, an additional short protected region (site II) spanning region –74 to –82 was detected. Promoter mapping experiments revealed that all three sites were of functional importance. Only the DNA fragment covering the region upstream of –14 permitted full promoter activity (fig. 2).

The protection that DegU offered to the *bacA* promoter at the three binding sites was relatively weak, but similar to the effect of DegU to the *bmy* promoter [Koumoutsi et al., 2007]; strong hypersensitive sites were observed

next to the binding sites. This implied that binding of DegU at either the *bacA* or the *bmyD* promoter rearranged the local DNA architecture, probably by inducing strong bending, constraint, or even unwinding, which makes the DNA more accessible to DNase I attack. In case of *bacA*, we propose that DegU binding at site I triggers a sharp DNA bend directly downstream from it (seen as hypersensitivity in footprinting analysis), thus enabling the DegU molecules sitting at the two upstream sites II and III to activate RNA polymerase. This mechanism has already been proposed for DegU activation of the *comK* and *bmyD* promoters [Hamoen et al., 2000; Koumoutsi et al., 2007]. The weak protection pattern of DegU was explained in case of the *bmyD* promoter by the nature of the DegU-binding sites [Koumoutsi et al., 2007]. Similar to *bmyD*, the binding sites of the *bacA* upstream region were A/T rich and appeared to be 'protected', even in the absence of their binding partner.

The *bacABCDE* operon and the monocistronic gene *ywfH*-encoding enzymes for the synthesis of bacilysin in *B. subtilis* [Steinborn et al., 2005] were organized similarly in both strains. It has been proposed that YwfH is also involved in bacilysin synthesis [Inaoka et al., 2003]. According to our EMSA and footprinting analysis, the *ywfH* promoter was directly activated by DegU, implying a common mechanism of gene activation in these members of the DegU regulon, the *bac* operon and *ywfH*. In contrast to the *bacA* promoter sequence, the *ywfH* promoter is less well conserved and direct affinity of the RNAP to the promoter sequence might be not sufficient for transcription. Three sites for DegU binding were identified in DNase I footprinting: –56 to –44, –37 to –14 and –4 to +18. However, in contrast to *bacA*, there was no indication for strong DNA bending during DegU binding, and only three bases were found to be hypersensitive to DNAse I digestion (fig. 5c). Thus, the effect of DegU to both promoters might be different. It is likely that DegU triggers RNAP binding at the *ywfH* promoter.

The role of ScoC as a negative regulator of *bacA* transcription in *B. subtilis* has already been established [Inaoka et al., 2009]. We found in our DNase I footprints that ScoC binding at the *bac* promoter sequence of FZB42 is tighter than that of DegU, and the areas of protections against DNase I digestion were much more pronounced (fig. 5b). However, preliminary competition experiments performed with both proteins indicated that under these conditions DegU bound more intensely than ScoC, but those data need further confirmation. In any case, interactions within the ternary complex formed by both regulators and the *bacA* promoter sequence seemed to be important for fine-tuning of transcription of the *bac* operon. In this context, it is interesting to note that regulation of the *ywfH* gene expression was also under control of the DegU global regulator protein.

Conclusion

Transcription of the *bac* operon and of the *ywfH* gene in *B. amyloliquefaciens* FZB42 was positively controlled by the DegU global regulator protein. The role of interactions within a ternary complex formed by the antagonistically acting regulators DegU and ScoC as well as the *bacA* promoter sequence remains to be elucidated.

Experimental Procedures

Strains, Plasmids, Growth Conditions and DNA Transformation

The strains and plasmids used in this study are listed in table 1. GA medium composed of 7 g/l K_2HPO_4, 2 g/l KH_2PO_4, 0.1 g/l $MgSO_4$, 1 g/l $(NH_4)_2SO_4$, 0.05 g/l yeast extract, 0.15 mg/l Fe $(SO_4)_3 \times 6\ H_2O$, 5 mg/l $MnSO_4 \times H_20$, 0.16 mg/l $CuSO_4 \times 5\ H_2O$ and 13 g/l sucrose was used for the cultivation of bacteria and antibiotic production. All plasmids were first transformed into Ca^{2+}-competent *E. coli* DH5α cells after ligation [Sambrook et al., 1989], and transformants were selected on LP-agar containing 100 μg/ml ampicillin. Plasmids and RNA were prepared using the NucleoSpin plasmid kit (Machery Nagel) according to manufacturer's protocols and confirmed by sequencing. Competent cells of *B. amyloliquefaciens* strains were prepared as described previously [Koumoutsi et al. 2007].

Primer Extension Analysis

Total RNA was isolated from FZB42 cells grown in GA medium. For the primer extension analysis, 40 ng of total RNA were mixed with 0.15 μM 5′-^{32}P-labeled primer, PeRW3 (*bacA)* or YwfH PE2 (*ywfH)*, respectively. Reverse transcription was carried out using M-MuLV reverse transcriptase (Fermentas) according to manufacturer's protocol. The reaction was stopped by adding loading buffer [95% deionized formamide, 20 mM EDTA (pH 8.0), 0.05% bromophenol blue and 0.05% xylencyanol]. The samples were denatured at 80°C and subsequently separated on a denaturating polyacrylamide sequencing gel (7 M urea, 6% polyacrylamide). Dideoxynucleotide sequencing reactions were performed using the Thermo Sequenase Cycle Sequencing kit (USB) with the same 5′-^{32}P-labeled primers.

Construction of Plasmids and Construction of B. amyloliquefaciens *FZB42 Mutant Strains*

The *scoC* mutant strain AM10 was constructed by amplifying a 2,561-bp PCR fragment covering the whole *scoC* gene and its flanking regions with the primer pair Hpr Fw and Hpr Rv (table 2). A central 573-bp fragment was removed by *Eco*47III and the Spec^r gene was inserted as a selective marker. The Spec^r cassette was constructed using spec FW and spec Rw primers (table 2).

Table 2. Primers used in this study

Primer	Sequence (59′ to 39′ end)/restriction site[1]	Use
Hpr FW	TGAAATAACCGCATACCGAAACC	AM10
Hpr Rv	TCGGATTCCTGGTCAATCAGAC	AM10
Amyback1fw	AAGAGTCCACATGGATGAGTG	pAK9
Amyfront2fw	TACAGCCATTCAGACATCTCC	pAK9
LacZfw	ACCAGACCAACTGGTAATGG	pAK9
LacZrv	TTGTTCCCACGGAGAATCC	pAK9
AK BacN1Fw-*Hin*dIII	ATATTAAAGCTTACGGCATGTATTCCTTTCTC	AA1
AKBacN1 Rv-*Sal*I	ATATTGTCGACGCACGATTCAAATGTATCATGC	AA1
AkBacN2Fw *Hin*dIII	TAATTAAGCTTAGCATCAGGCCGACCAAAC	AA2
AkBacN3Fw *Hin*dIII	ATTAAAGCTTAAATGTTAATTTTAACACCC	AA3
AkBacN4Fw *Hin*dIII	TTATTAAGCTTTGTTTTCTAATATATAGG	AA4
AkBacN5Fw *Hin*dIII	TTTATAAGCTTTGACAGCTTGAACATCTATG	AA5
bacFP1a	ATGAGCATCAGGCCGACCAAAC	FP/EMSA
bacFP1b	ATCCATAGATGTTCAAGCTGTC	FP
bacFP2a	TCATAGGGTGTTTTCTAATATATAG	FP
bacFP2b	TTAAGTAAATATTATCCATAGATG	FP
bacFP3a	TTCAAATAATATTGACAGCTTG	FP
bacFP3b	TGATCGTAATCAGTTTAGATATTG	FP/EMSA
PeRW3	ACTACTTGTCCCTTCAGGACCG	PE
YwfH PE 2	ATAATAAACGCGGTTCGTTTTGAC	PE
ywfH-*Hin*dIII	ATATATAAGCTTAGCGATGATGTGCTTCAGTTC	AA6
ywfH-*Sal*I	ATATTGTCGACTTCTTTCCATAGGTTTCCGACG	AA6
ywfH Fw	TCGACACGCTTCCGAAGTTTTTG	FP
ywfH Rv	ACGCGGTTCGTTTTGACAACTG	FP
ywfH Rv *Nde*I	TTAATACATATGAATCGTGTTGAACCGCC	
Hpr2Rw *Bam*HI	ATTAATGGATCCTTTATTGAGATTATGAAGCAC	
Spec Fw	CTCAGTGGAACGAAAACTCACG	AM10
Spec Rv	TAAGGTGGATACACATCTTGTC	AM10

[1] Restriction sites are underlined.

Chromosomal DNAs isolated from strains CH23, TF1 [Koumoutsi et al., 2007] or AM10 (*scoC*::Spec^r) were used in transformation of AA2 and AA6 yielding mutants AM11 (*comA*::Em^r), AM12 (*degU*::Em^r), AM13 (*scoC*::Em^r) and AM14 (*degU*::Em^r; table 1).

Construction of bacA::lacZ *and* ywfH::lacZ *Transcriptional Fusions in* B. amyloliquefaciens *FZB42*

Vector pAK9 [Koumoutsi et al., 2007] was used to integrate single copy transcriptional fusions at the *amyE* locus of the FZB42 chromosome via double cross-over recombination. To analyze the promoter activity of *bacA,* DNA fragments of various lengths bearing the *bacA* promoter region were amplified using BacN1, BaN2, BacN3, BacN4 and BacN5 as forward and BacN1Rv as reverse primers (table 2). Resulting PCR-products were ligated upstream of the *lacZ* reporter gene into the *Hin*dIII and *Sal*I site of pAK9 yielding plasmids pAFN1 to pAFN5 (table 1). Transcriptional fusion of the *ywfH* promoter with *lacZ* reporter gene was prepared in the same manner using primers YwfH Fw and YwfH Rv for amplification. The PCR product was inserted into the *Hin*dIII and *Sal*I site of pAK9 yielding the plasmid pAYN1 (table 1). The linearized plasmids were finally transformed into competent *B. amyloliquefaciens* AA1 cells (table 1) as single copy at the *amyE* locus. The integrated transcriptional fusion was corroborated by PCR and the resulting strains were named AA2 to AA6 according to plasmids pANF1 to pANF5. Strain AA6 harbored plasmid pAYN1 (table 1). The mutant strains AM11, AM12 and AM13 (table 1) were prepared using transformation of strain AA2 with chromosomal DNA isolated from the mutant strains CH23 (Δ*comA*), TF1 (Δ*degU*) and AM10 (Δ*scoC*).

To obtain the expression plasmid pHPR, the coding site of *scoC* was amplified using primer pair Hpr2Fw NdeI/ Hpr2Rw *Bam*HI (table 2). The PCR product was inserted into the *Nde*I/*Bam*HI site of pET15b (Novagen), which carries an N-terminal His tag.

β-Galactosidase Assay

Specific β-galactosidase activity was determined from growing liquid cultures in GA medium. β-Galactosidase was assayed using o-nitrophenyl-β-D-galactopyranoside as the substrate, and is reported in Miller units [Miller, 1972].

Purification of DegU and Hpr

The DegU protein was prepared as described previously [Koumoutsi et al., 2007]. The ScoC protein was overexpressed in *E. coli* BL21 (DE3) harboring expression plasmid pHPR as an overnight culture in LB containing 1% lactose. Cell lysis and protein purification was performed as described previously for AbrB [Makarewicz et al., 2008]. Phosphorylated DegU was prepared after incubating DegU with acetyl phosphate (1 mM) in phosphorylation buffer [20 mM Tris-HCl (pH 8), 100 mM KCl, 5 mM MgC_2, 0.5 mM dithiothreitol, and 10% glycerol] at 37°C for 1 h. The degree of phosphorylation of the purified protein was checked by SDS gel electrophoresis.

Electrophoretic Mobility Shift Assay

A 264-bp DNA fragment harboring the *bacA* promoter region between bp –171 and +93 relative to the translational start was amplified using primers bacFP1a (5′-^{32}P-labeled) and bacFP3b. 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 MgC_2, 0.5 mM dithiothreitol, 10% glycerol, 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 nondenaturating gels (6% polyacrylamide) in 1 × Tris-borate-EDTA buffer (pH 8) at 60 V.

Similarly, the same fragment containing the promoter region between bp –171 and +93 relative to the translational start was incubated with different amounts of purified ScoC at 37°C for 20 min, under the same conditions and buffers used for DegU.

In the case of *ywfH*, a fragment (170 bp) synthesized through primer ywfH fwFP and ywfH rwFP was radio-labeled, purified and incubated with increasing concentrations of DegU at 37°C for 20 min. The mixture was then separated using nondenaturating 6% polyacrylamide gel electrophoresis.

DNase I Footprinting Assay

The 271-bp fragment of the *bacA* promoter (–671 to +221) was amplified using the primers bacFP1a and bacFP3b and the 180-bp DNA fragment (–81 to +221) was obtained using primers bacFP2a and bacFP3b. A 150-bp fragment of the *ywfH* promoter (–119 to +39) were amplified using primers ywfH Fw-FP and ywfH Rw-FP. Since one primer was radio-labeled, the corresponding fragments were labelled either at the coding or at the noncoding strand.

DNase I footprinting was performed as described previously [Derre et al., 1990]. DNA was incubated in binding buffer with different amounts of DegU protein (0–10 μM) or ScoC (0–8 μM) for 30 min at 37°C. Complexes were then treated with DNase I for 1 min, and the reaction was stopped by addition of DNase I stop solution (0.4 M sodium acetate, 50 μg/ml calf thymus-DNA, 2.5 mM EDTA). The treated complex was precipitated using 95% ethanol and dried at 50°C, then the DNA was resuspended in 5 μl of loading buffer, denatured at 80°C and separated on 7 M urea and 6% polyacrylamide sequencing gels and visualized using the Molecular Imager FXpro Plus (Bio-Rad).

Bioautography Assay

Bioautography was performed as previously described [Chen et al., 2006; Hofemeister et al., 2004]. Twenty-microliter samples derived from supernatants from bacteria grown overnight in GA medium were loaded onto the silica gel 60 F254 thin layer chromatography (TLC) aluminium sheets (20 × 20 cm; Merck, Darmstadt, Germany), and separated by TLC chromatography [1-butanol/acetic acid/water 4:1:1 (v/v/v)]. Dried chromatographic plates were placed for 2 h onto agar cultures prepared with *B. megaterium* as an indicator stain to test bacilysin and difficidin production. Then they were incubated overnight at 37°C. Zones of incubation documented the positions of antibiotics separated by TLC.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of FZB42 *bacA*, *ywfH* and *scoC* have been published [Chen et al., 2007]. They are available from the complete genome sequence of FZB42, which is available under GenBank accession No. CP000560.

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