J. Microbiol. Biotechnol. (2010), **20**(2), 301–310
doi: 10.4014/jmb.0906.06025
First published online 30 December 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Three Non-Aspartate Amino Acid Mutations in the ComA Response Regulator Receiver Motif Severely Decrease Surfactin Production, Competence Development, and Spore Formation in *Bacillus subtilis*

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Received: June 12, 2009 / Revised: September 16, 2009 / Accepted: October 1, 2009

Bacillus subtilis **strains produce a broad spectrum of bioactive peptides. The lipopeptide surfactin belongs to one well-known class, which includes amphiphilic membrane-active biosurfactants and peptide antibiotics. Both the *srfA* promoter and the ComP–ComA signal transduction system are an important part of the factor that results in the production of surfactin. Bs-M49, obtained by means of low-energy ion implantation in wild-type Bs-916, produced significantly lower levels of surfactin, and had no obvious effects against *R. solani*. Occasionally, we found strain Bs-M49 decreased spore formation and the development of competence. Blast comparison of the sequences from Bs-916 and M49 indicate that there is no difference in the *srfA* operon promoter P*srfA*, but there are differences in the coding sequence of the *comA* gene. These differences result in three missense mutations within the M49 ComA protein. RT–PCR analyses results showed that the expression levels of selected genes involved in competence and sporulation in both the wild-type Bs-916 and mutant M49 strains were significantly different. When we integrated the *comA* ORF into the chromosome of M49 at the *amyE* locus, M49 restored hemolytic activity and antifungal activity. Then, HPLC analyses results also showed the *comA*-complemented strain had a similar ability to produce surfactin with wild-type strain Bs-916. These data suggested that the mutation of three key amino acids in ComA greatly affected the biological activity of *Bacillus subtilis*. ComA protein 3D structure prediction and motif search prediction indicated that ComA has two obvious motifs common to response regulator proteins, which are the N-terminal response regulator receiver motif and the C-terminal helix-turn-helix motif. The three residues in the ComA N-terminal portion may be involved in phosphorylation activation mechanism. These structural prediction results implicate that three mutated residues in the ComA protein may play an important role in the formation of a salt-bridge to the phosphoryl group keeping active conformation to subsequent regulation of the expression of downstream genes.**

Keywords: Surfactin production, phosphorylation, two-component regulatory system, antifungal activity

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Bacillus subtilis strains produce a broad spectrum of bioactive peptides that have great potential for biotechnological and biopharmaceutical applications. One well-known class is composed of amphiphilic membrane-active biosurfactants and peptide antibiotics with potent antimicrobial activities. Examples of lipopeptides from this class include surfactin [3, 21, 24], fengycin [50], and the iturin compounds [6]. Surfactin is an extracellular peptide antibiotic, and its production is well understood. The *srfA* locus is required for the production of surfactin [33] and is an operon spanning over 25 kb. The *srfA* locus has been shown to have an important function in cell specialization and differentiation [32]. The *srfA* expression is induced after the end of exponential growth and is dependent on the products of late-growth regulatory genes *comp* and *comA*, as well as the *srfA* promoter region [34]. There is a region of dyad symmetry found upstream of the promoter that may be the target for ComA-dependent transcriptional activation.

Another important part of the surfactin pathway is the ComP–ComA signal transduction system. ComP and ComA are believed to be partners in a two-component regulatory system [8, 19] and to play a role in sensing the nutritional environment. ComP is a histidine kinase, which contains a membrane-spanning domain and serves as the sensor class of two-component proteins [53]. ComA is a response regulator protein and contains three highly conserved aspartic acid residues in the N-terminal portion of the protein.

These conserved residues may be the targets for ComP-catalyzed phosphorylation [19, 52]. ComA also contains a possible helix-turn-helix domain, which suggests that ComA may also be a DNA-binding protein [52].

The ComP–ComA system is thought to be a major quorum response pathway in *B. subtilis*. This system also regulates the development of genetic competence [17], along with other protein partners including ComX and ComQ. The ComX pheromone is a farnesylated 10-amino-acid peptide that is secreted and accumulates outside of the cell [2, 29, 34]. It can activate ComP, causing ComP to autophosphorylate and, subsequently, donate a phosphate group to ComA. CSF (also known as PhrC) is a 5-amino-acid peptide that contributes to the activation of ComA by inhibiting the activity of the regulator RapC [13, 43]. Once phosphorylated and active, ComA functions as a transcriptional activator. Production of the active ComX pheromone requires *comQ* [29, 40], and together, the *comQ–comX–comP–comA* gene cluster is a peptide signaling cassette similar to those found in many other Gram-positive bacteria [25]. The contribution of the ComX–ComP–ComA signaling pathway to competence development is well understood [46]. *comQ*, *comX*, *comP*, and *comA* were named for their roles in competence development, and a null mutation in any of these genes causes a decrease in competence development. The only known direct link between quorum sensing and competence is a small gene, *comS*, that is embedded in the large *srfA* operon [20, 44]. ComA directly activates transcription of the *srfA* operon [34, 35, 38, 49], including *comS*. Once produced, ComS prevents the degradation of the competence transcription factor ComK [47], thereby affecting expression of over 100 genes, including those encoding the transformation machinery [5, 36].

Many genes that are directly regulated by the transcription factor ComA have been identified, including *degQ*, which encodes a regulator of degradative enzyme production [30] and the regulatory proteins *rapA* [31], *rapC* [43], *rapE* [23], and *rapF* [22]. Recently, it was shown that overexpression of ComA in cells in stationary phase induced the *srfA* operon and *rapA*, both known targets of ComA, at least 4.5-fold [37].

Interestingly, ComA, ComP, and ComX affect the same set of genes, indicating that the kinase ComP is the only receptor for the signaling molecule ComX, and that ComA is the only transcription factor activated directly by ComP [12]. The binding site for ComA has been defined by *in vitro* binding studies, including gel shift and footprinting experiments, combined with mutational analyses [35, 38]. The consensus sequence is a 12 bp palindrome (TTGCGG-$N_{4/5}$-CCGCAA) with a 4 or 5 bp spacer. Genes known to be directly activated by ComA contain at least one binding site, and searches of the *B. subtilis* genome have identified many potential target genes that contain candidate binding sites.

Bacillus subtilis 916 was an effective biocontrol agent against the rice sheath blight caused by *Rhizoctonia solani* and was utilized in agriculture for its ability to promote plant growth and to suppress plant pathogenic organisms [9]. Previously, we demonstrated that the mutant strain Bs-M49, obtained by means of low-energy ion implantation in wild-type Bs-916 [The low-energy ion implantation involved not only a combination of energy deposition (similar to λ-ray radiation) but also a mass deposition and charge exchange of energetic ions.], which produces significantly lower levels of surfactin, had no obvious effects against *R. solani*. Thin-layer chromatography and high performance liquid chromatography (HPLC) analyses indicated that the lipopeptides produced by Bs-916 belonged to the surfactin family. These results suggested that the surfactin produced by Bs-916 plays an important role in the suppression of sheath blight [28]. In addition, we occasionally observed that strain Bs-M49 showed abnormal phenotypes in sporulation and the development of competence. Further research was carried out to identify the relationship between these phenotypes and the reduced levels of surfactin in *Bacillus subtilis*. In this study, we also demonstrated that surfactins were the key antifungal substance for Bs-916, and that this involved an interesting molecular mechanism. These results may lead to a better understanding of how a reduction in surfactin production can affect the ability of a strain to serve as a biocontrol agent.

Materials and Methods

Strain and Plasmid

Bacterial strain DH5α was purchased from Tiangen Ltd (Beijing, China). *B. subtilis* strain Bs-916 was obtained from our laboratory. The integration vectors pMUTIN4 [48] and pDG1728 [18] were obtained from BGSC (Table 1).

Culture Media

Luria–Bertani (LB) medium was made as previously described [39]. *B. subtilis* strains were grown at 28°C in either YPGA medium or YPG liquid medium with an agitation rate of 130 rpm.

DNA Manipulation

Plasmids were transformed in *Bacillus subtilis* as previously described, with reference to general molecular biology methods for *Bacillus* [14]. Transformed cells were washed in SP buffer (0.15 M NaCl, 0.02 M K_2HPO_4, pH 7.5). The washed samples were resuspended in lysis medium (0.05 M NaCl, 0.1 M EDTA, pH 6.9). SPII medium contained Spizizen salts, 0.02% casamino acids, 0.1% Difco yeast extract, 0.5% glucose, 0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, and 50 μg/ml of each required amino acid. Competent cells were prepared and frozen. Frozen competent cells were quickly thawed and incubated for 5 min at 37°C. To begin the reaction, DNA was then added to a final concentration of 1–2 μg/ml. The mixture was incubated at 37°C for 7 min or 9 min, at which time an excess (100 μg/ml) of EGTA was added to stop further DNA uptake. Transformants were

Table 1. Strains and plasmids.

Strain or plasmid	Characteristics	Source
Strains		
Escherichia coli		
DH5α	F^- Φ80d*lacZ* ΔM12 *minirecA*	Our lab.
Bacillus subtilis 916	Wild type	Our lab.
Bacillus subtilis M49	Surfactin-decreased mutant	Our lab.
Bacillus subtilis M49C3	*Spec*^{r} transformant; pDCOMA plasmid is transformed into M49 strain, and allowed integration of *comA* ORF into the chromosome of M49 at *amyE* locus	This study
Bacillus subtilis MC1	*Erm*^{r} transformant; pMCOMA plasmid is transformed into wild-type strain	This study
Plasmids		
pMD18-T	T-clone site vector; *Amp*^{r}	Takara
pMUTIN4	*spoVG-lacZ*; *Erm*^{r}; *Amp*^{r}	BGSC
pDG1728	*amyE*'... '*amyE* 5' and 3' segments of the *B. subtilis* 168 *amyE* gene; *spoVG-lacZ*; *Spec*^{r}; *Erm*^{r}; *Amp*^{r}	BGSC
pDCOMA	642 bp *Hin*dIII–*Bam*HI *comA* fragment joined to *Hin*dIII–*Bam*HI restricted pDG1728	This study
pMCOMA	639 bp *Hin*dIII–*Bam*HI *comA* fragment joined to *Hin*dIII–*Bam*HI restricted pMUTIN4	This study

Amp^r: ampicillin resistance; Km^r: kanamycin resistance; Erm^r: erythromycin resistance; Spec^r: spectinomycin resistance.

selected on LB plates containing 100 μg/ml spectinomycin. To calculate the transformation competency of *B. subtilis* cells, the number of colonies on the plate was divided by the amount of DNA (in μg) added to the transformation. The results are expressed in number of transformants (or colony forming units) per microgram of plasmid DNA (CFU/μg). A quantity of 1 μg/ml pDG1728 plasmid was used for DNA transformation.

Measurement of Sporulation

Strains to be tested for sporulation were grown for 24 h in nutrient sporulation medium [33] at 37°C and plated for viable counts before and after incubation of the viable count dilution tubes at 80°C for 20 min.

Construction of Mutants

A fragment of the target genes was amplified from Bs-916 chromosomal DNA by PCR with primers (Table 1) and then extracted from polyacrylamide gels after electrophoresis. Both products and vectors (pMUTIN4 and pDG1728) were digested with *Hin*dIII and *Bam*HI. Digested fragments and plasmid were then mixed for ligation. The ligation mixtures were used to transform *E. coli* competent cells. Recombinant plasmids were purified and then used to transform *B. subtilis* competent cells. Transformants were selected on LB plates containing 0.3 μg/ml erythromycin or 100 μg/ml spectinomycin. HPLC analysis was used to confirm the transformants.

RT–PCR

Semiquantitative RT–PCR was performed to determine the transcriptional expression of the *rapA*, *rapC*, and *comS* genes. RNA was extracted with a RNAprep pure kit (Tiangen Ltd, Beijing, China). One to two μl of RNA solution was digested with DNase I (TaKaRa Biotech. Co. Ltd, Dalian, China) and used in the RT–PCR analyses. RT was initiated in the presence of oligo-(dT) primers (42°C, 30 min), and after inactivation of the reverse transcriptase (95°C, 5 min), the appropriate primers (Table 2) were added for PCR cycling (30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C). Amplification of a constitutively expressed gene (*16s RNA*) served as an internal control in the RT–PCR assays.

Extraction and Analysis of Surfactin Produced by *B. subtilis* Strains

Bs-916 was incubated for 36 h in an YPG medium at 28°C in a shaker. Subsequently, 1 ml of the culture broth was inoculated into 300 ml of YPG medium in a 1,000-ml Erlenmeyer flask. The flask was put into a shaking incubator for 36 h at 28°C. The fermentation broth was separated by centrifugation (8,000 rpm, 20 min), and bacterial cells were discarded. The culture supernatant was collected and adjusted to pH 2.0 with 12 N HCl. The precipitation formed at 4°C

Table 2. Oligonucleotide sequences of competence- and sporulation-associated genes.

Oligonucleotide	Oligonucleotide sequences
comA	5'-ATGAAAAAGATACTAGTGATTGATG-3' 5'-TTAAAGTACACCGTCTGATTTC-3'
P*srfA*	5'-AAAATGTCATGAAAGAATCGTTGT AAG-3' 5'-CGCAAGATTTGAAATGCTCGTGTGA-3'
pDG1728- *comA*	5'-TTAGAATTCGGGGCTTTCTGG-3' 5'-TATGGATCCTTAAAGTACACCGTCT-3'
pMUTIN4- *comA*	5'-CGAAGCTTATGAAAAAGATACTAG TGATT-3' 5'-TCGGATCCAAGTACACCGTCTGATT-3'
comS	5'-AAGCATCTTATCAGCAGCAT-3' 5'-TATCTACTTCTCCCTCCAGCA-3'
rapA	5'-TTCCGCATTCTATCTACTCTT-3' 5'-TTTCGGGCTGTCCGTTTT-3'
rapC	5'-CCTTTGTCGCAGACCATA-3' 5'-ATAGCATAACCCGAGATT-3'
16s RNA	5'-GCCTGTAAGACTGGGATAACT-3' 5'-GACAACGCTTGCCACCTA-3'

overnight and the precipitate was collected by centrifugation and extracted with methanol. The extract was filtered through a 0.2-μm pore-size polytetrafluoroethylene membrane (JP020; Advantec, Tokyo, Japan) and injected into a reverse-phase high-performance liquid chromatography (HPLC) column (column, 5RPC; 2.1 mm in diameter×150 mm; GE Sciences, U.S.A.). The system was operated at a flow rate of 0.2 ml/min and monitored at 215 nm with the solvent 3.8 mM acetonitrile:trifluoracetic acid [80:20 (v/v)]. The concentration of surfactin was determined with the standard surfactin purchased from Sigma. UNICORN software (GE Sciences, U.S.A.) was used to identify and quantify the results.

Surfactin Production Assay

Surfactin production was assayed on blood agar plates made by pouring 5 ml of minimal medium (MM) soft agar overlay supplemented with 0.5 ml of bovine blood onto MM agar with nutritional requirements. To make MM agar, 10 ml of 1.2% $MgSO_4$, 10 ml of 50% glucose, 100 ml of l0× T base [20 g of $(NH_4)_2SO_4$, 140 g of K_2HPO_4, 60 g of KH_2PO_4, and 10 g of sodium citrate per liter], and 20 ml of 0.5% glutamate were added to 1 liter of 1.2% agar after autoclaving. The fermentation broth was separated by centrifugation (8,000 rpm, 20 min), and bacterial cells were discarded. The culture supernatant was collected after autoclaving. Two hundred μl portions of supernatant samples were deposited in 10-mm-diameter wells created in the solidified media using sterile glass tubes. The plates were incubated at either 30°C or 37°C depending on the strain tested.

Antifungal Activities Test

R. solani agar-screening plate was used to test for antifungal activity. Indicator lawns were made by incubating *R. solani* on PDA Petri disks. The screening plates were obtained by preparing 5-mm pats from *R. solani* lawns plates with a sterilized borer and placed in the center of the PDA plate. The bacterial colonies were picked and incubated on the screening plate. The plates were incubated at 28°C for 36 h, and inhibitory activity was quantified by the length between the edges of the Petri disk and the indicator strain. The wild-type *B. subtilis* strain was used as a control of these experiments. The results presented are averages of three independent experiments.

ComA Protein 3D Structure Prediction and Motif Search Prediction

ComA protein 3D structure was obtained from the CBS (Center for Biological Sequence analysis, http://www.cbs.dtu.dk/services/CPHmodels/) Web site. We used the Nettest program, a novel method, to search for fold recognition and homology. Nettest iteratively searches large sequence databases to construct a sequence profile until a template is found in a database of proteins with known structure. The method differs from the PDB–BLAST method in that a sequence profile is only made if a template is not readily found in the database of known structures. The ComA protein structure was based on the templates of the *Mycobacterium tuberculosis* hypoxic response regulator DosR (PDB ID 3C3W) and the LuxR protein (PDB ID 3CZ5). The corresponding atoms derived from the alignment can be extracted from the template file and used as a starting point for the homology modeling. Missing atoms were added using the Segmod program [26] from the GeneMine package (http://www.bioinformatics.ucla.edu/genemine/). The structures can then be refined using the Encad program [27] from the GeneMine package. The 3D structure was viewed and analyzed using Discovery Studio Visualizer software (Accelrys Ltd). Motif search results were obtained from NPSA web site servers (http://npsa-pbil.ibcp.fr).

RESULTS

M49 Confers a Leaky Competence and Sporulation-Deficient Phenotype

The defect in *comA* in *B. subtilis* strain M49 confers a leaky competence phenotype, typified by a 10-fold reduction. This indicated the fact that DNA fragments are more easily transformed to wild-type strain than M49 mutant.

The M49 strain also appeared to exhibit a sporulation-deficient phenotype on solid media. We followed up this observation by testing the sporulation frequency of both the M49 and wild-type strains in liquid media. The Bs-916 strain yielded 2.1×10^8 viable CFU, of which 2×10^8 (91%)

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B-916       MKKILVIDDHPAVMEGTKTILETDSNLSVDCLSPEPSEQFIKQHDFSSYDLILMDLNLGG 60
M49         MKKILVIDDHPAVMEGTKTILETDSNLSVDCLSPEPSEQFIKQHDFSSYDLILMDLNLGG 60
            ************************************************************

B-916       EVNGMELSKQILQENPHCKIIVYTGYEVEDYFEEAIRAGLHGAISKTESKEKITQYIYHV 120
M49         EVNGMELSKQILQENPHCKIIVYTGYEVEDYFEEAIRAGLHGAISNTESKEKITQYIYHV 120
            ************************************************:***********

B-916       LNGEILVDFAYFKQLMTQQKIKPAPSSQKEQDVLTPRECLILQEVEKGFTNQEIADALHL 180
M49         LNGEILVDFAYFKQLMTQQKFKPAPSSQKEQDVLTPRECLILQEVEKGFTNQEIADALHL 180
            **** ***************.****************************************

B-916       SKRSIEYSLTSIFNKLNVGSRTEAVLIAKSDGVL 214
M49         SKRSIEYSLTSIFNKLNVGSRTEAVLIAKSDGVL 214
            **********************************
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Fig. 1. Amino acid sequence analysis of Bs-916 and mutant M49 strains.
Amino acid residues that are identical (*), conserved (:), similar (.), and different (boldface italic) between the wild-type strain and M49 are all indicated. The predicted helix-turn-helix motif is depicted as an open rectangle, and conserved residues that are reported to be involved in the phosphorylation activation mechanism are depicted as gray rectangles. K106 is also thought to be involved in the phosphorylation activation mechanism.

were heat resistant. The M49 strain yielded 2.2×10^8 CFU, of which 1.2×10^7 (5.5%) were heat resistant. This confirmed the observation based on colony growth on solid media and indicated that the M49 strain did have a sporulation-deficient phenotype.

comA Gene and *srfA* Promoter Sequence Analysis

The *srfA* promoter (P*srfA*) and *comA* sequences were aligned by the ClustW program. The *comA* gene and P*srfA* were amplified from M49 and Bs-916 utilizing primers listed in Table 2. Our data showed that three residues were substituted in M49's ComA protein, at the 106th, 125th, and 14st amino acid sites (Fig. 1). The P*srfA* sequence was 100% conserved between the M49 and Bs-916 strains (data not shown). These results suggested that reduction in surfactin production may be due to the mutation of a few key amino acids rather than to a difference in the operon promoter P*srfA*. These mutations could have a significant effect on the activity of the ComA protein.

RNA Analyses

RT–PCR was used to determine the expression levels of selected genes involved in competence and sporulation in both the wild-type Bs-916 and mutant M49 strains. Both the *comS* and *rapC* genes were expressed in the Bs-916 strain but not in the M49 strain. The *rapA* gene was expressed at a similar level in both strains (Fig. 2).

Surfactin Production Assay and Antifungal Activity Test

Surfactin production was assayed on blood agar plates. The hemolytic activities of Bs-916 (wild-type strain), MC1 (*comA* knockout mutant), M49C3 (pDCOMA plasmid transformed into M49 strain, and allowed to integrate *comA* ORF into the chromosome of M49 at *amyE* locus), and M49 mutant were tested. Both MC1 and M49 had minimal hemolytic activity. Both M49C3 and Bs-916 had similar levels of hemolytic activity (Fig. 3).

The antifungal properties of all the strains against *R. solani* were analyzed. As in the hemolytic activity assay, both the *comA*-deficient mutants, MC1 and M49, had a growth inhibition zone that was significantly smaller than strains M49C3 and Bs-916 (Fig. 4).

HPLC Analysis of Surfactin Production

Surfactin production of each of the four strains was determined by HPLC (Fig. 5). Extract from liquid cultures

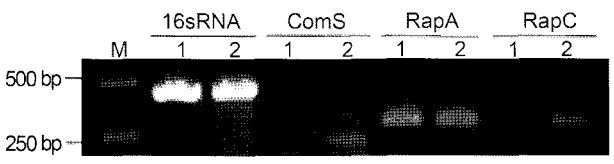

Fig. 2. Expression of *comS*, *rapA*, and *rapC* genes in M49 and Bs-916. M, DL2000; 1, M49; 2, Bs-916.

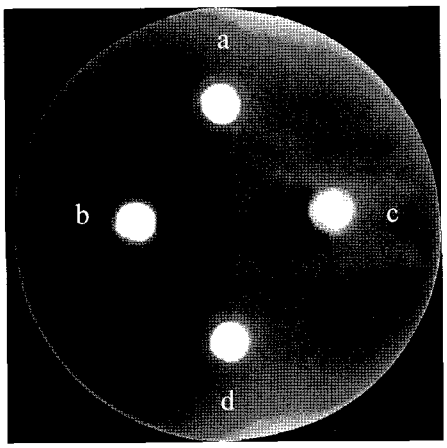

Fig. 3. Hemolytic activity of wild-type Bs-916 strain (a), mutant M49 strain (b), *comA* knockout MC1 strain (c) and *comA* complement M49C3 strain (d) in MM blood medium.

was filtered through a 0.2-μm polytetrafluoroethylene membrane and injected into a reverse-phase HPLC column. Commercially purchased surfactin was used as a standard control. Based on retention time, the peaks from each of the strains were shown to correspond to the surfactin standard control. As previously reported [28], the M49 strain exhibited an impaired capacity to produce surfactin. MC1 also had a significant reduction of its ability to produce surfactin. However, both Bs-916 and M49C3 produced surfactin at wild-type levels (Fig. 5).

ComA Protein 3D Structure Prediction and Motif Search Prediction

The ComA protein 3D structure was predicted using several modeling programs. First, LuxR (PDB ID 3CZ5) and chain A of *Mycobacterium tuberculosis* hypoxic response regulator Dosr (crystal structure 3C3W) [55] were used as modeling templates. After generation of the homology

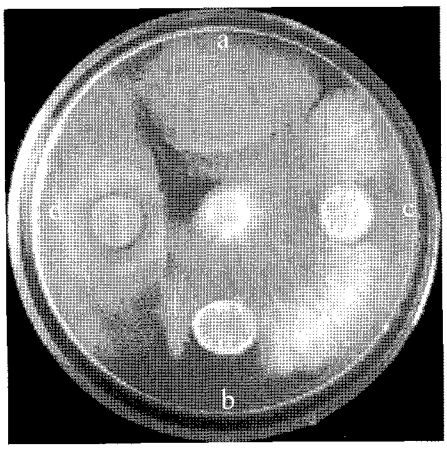

Fig. 4. Antifungal activity of wild-type Bs-916 strain (a), mutant M49 strain (b), *comA* knockout MC1 strain (c) and *comA* complement M49C3 strain (d).

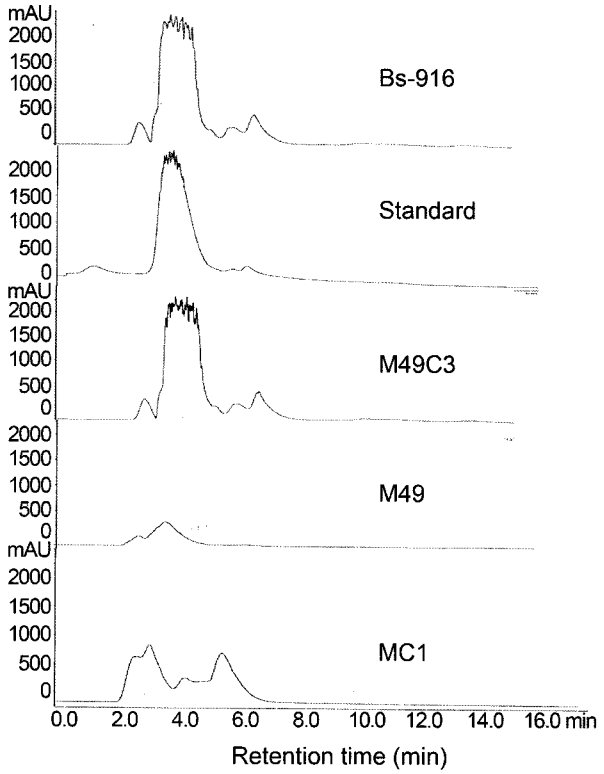

Fig. 5. Detection of surfactin by analytical reverse-phase HPLC in wild-type and mutant strains (detection at 215 nm).

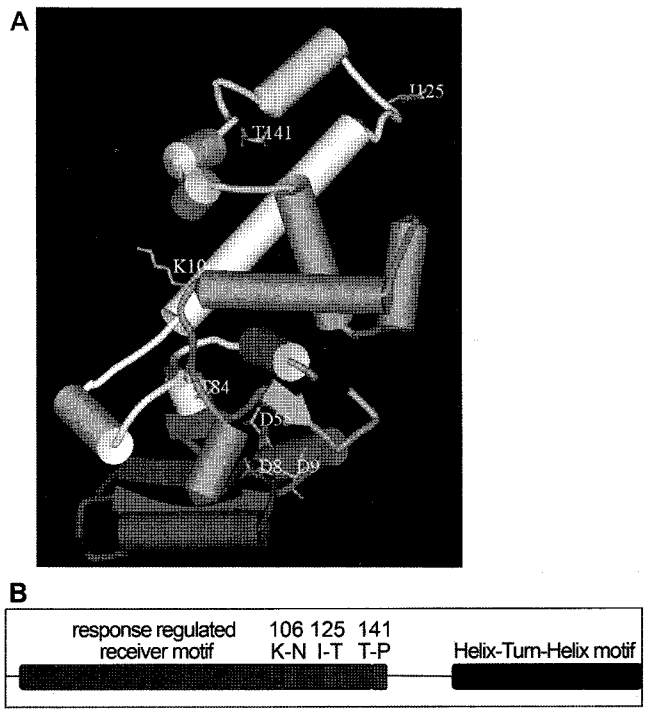

Fig. 6. Predicted phosphorylation sites (**A**) and conserved motifs (**B**) in ComA.
N-terminal domain sites include the residues D8, D9, D55, and T84, and loop linker sites include the residues K106, I125, and T141. The N-terminal region is shown in blue. The C-terminal region is shown in red. **B.** The response regulator receiver motif is depicted as a green rectangle, and the HTH motif is depicted as a brown rectangle. The missense amino acids changes found in M49 are depicted as red rectangles.

model, the 3D structure of ComA was then refined using the Encad program. The 3D structure includes 12 helices and 5 sheets (Fig. 6). Conserved residues thought to be involved in the phosphorylation activation mechanism are labeled on the 3D structure. Five residues (D8, D9, D55, T84, and K106) were previously reported [55], and the other two residues (I125 and T141) were identified in this study. The amino acid residues K106, I125, and T141 were determined to be located within the loop linker region (Fig. 6A). Motif search results revealed two predicted motifs in the ComA protein. One is an N-terminal response regulator receiver motif, and the other is a C-terminal helix-turn-helix (HTH) motif (Fig. 6B).

Discussion

ComA Protein Missense Mutations, But Not the *srfA* Promoter, are Responsible for Leaky Competence- and Sporulation-Deficient Phenotypes of the M49 Strain

We demonstrated that the M49 strain is deficient in both competence and sporulation. BLAST comparison of the sequences from Bs-916 and M49 indicate that there is no difference in the *srfA* operon promoter P*srfA*, but there are differences in the coding sequence of the *comA* gene. These differences result in three missense mutations within the M49 ComA protein. These results indicate that the M49 leaky competence and sporulation-deficient phenotypes involve the *comA* gene.

ComA Mutant Seriously Decreases Expression of Competence- and Sporulation-Associated Genes

ComA is involved in competence development, sporulation, and degradative enzyme production [17]. Both ComA and ComP are also needed for the regulated expression of *degQ, rapA*, and *rapC*. These genes encode products that are expressed in response to nutrient starvation but are not required for competence [12]. In Bs-916, ComA stimulates the expression of *rapA* and *rapC*. However, the transcription of *rapC* is not activated in M49. In addition, the competence regulatory factor ComS, which lies within and out-of-frame with the *srfA*B gene, was active in the wild-type strain and inactive in the *comA*-deficient mutant. Since these genes, all of which are involved in competence and sporulation, are directly regulated by *comA*, their transcription levels are affected by the missense mutations in the ComA protein. These results can be used to explain the reductions in competence, spore formation, and surfactin production in the M49 strain.

Production of Surfactin is Essential for Bs-916 as a Biocontrol Agent

ComA is also required for the expression of *srfA*, an operon involved in the production of the peptide antibiotic surfactin [34]. Qualitative and quantitative analysis of the crude extracts from Bs-916 and M49 demonstrated that the lipopeptides produced by these strains belonged to the surfactin family [28]. To further verify the role of surfactin in the biocontrol of *R. solani*, we obtained a *comA*-restored mutant of Bs-916, strain M49C3, which showed a similar antagonistic ability against *R. solani* as well as wild-type production levels of surfactin. We also demonstrated that surfactins were key antifungal substances. These findings strongly suggest that the production of surfactins is a crucial element in the ability of Bs-916 to act as a biocontrol agent.

Three ComA N-Terminal Residues May Play a Key Role in the Induction of Protein Conformation Changes Following Phosphorylation

How phosphorylation activates response regulators is one of the key questions in two-component regulatory system research. Response regulators are believed to be in equilibrium between inactive and active conformations, with phosphorylation acting as a molecular switch to shift this equilibrium towards the active form [1, 11, 41, 51]. In all activated structures of the N-terminal regulatory domain, phosphorylation (or BeF_3^- activation) of a critical Asp residue causes movement of a conserved Lys (K106 in the ComA protein) in the β5–α5 loop to form a salt-bridge to the phosphoryl group. Phosphorylation also causes a conformational change of a conserved Thr residue in strand β4 to form a hydrogen bond with a phosphoryl oxygen atom. This allows the side chain of a conserved Tyr residue in β5 to reorient from a solvent-exposed outward position to fill a pocket vacated by the Thr residue in a buried inward position with concomitant conformational changes of surrounding residues [11].

Typical two-component regulatory systems contain a membrane-bound sensor kinase, which has a crucial role in sensing environmental stimuli, and a cytosolic response regulator, which usually consists of an N-terminal domain, a linker and a C-terminal domain. The ComA N-terminal sequence shows similarity to many of the response regulator proteins and contains several amino acid residues that are invariant in these proteins [8]. In particular, it has a conserved aspartate residue (D55), which is a likely site for phosphorylation [52]. In addition, the C-terminal region of the predicted ComA sequence contains a possible helix-turn-helix motif, which resembles the DNA binding sequences of a family of transcriptionally active proteins. Roggianit and Dubnau [38] determined that phospho-ComA binds directly to the promoter region of *srfA in vitro* and that this protein binds to sites on the DNA corresponding to the previously identified ComA boxes. In the absence of protein 3D structures, the mechanism responsible for the interaction between ComA and ComP remains a puzzle.

In this study, we identified five conserved residues in ComA, which had previously been reported to be involved in the phosphorylation activation mechanism. These residues include three Asp (Asp8, Asp9, and Asp55), one Thr/Ser (Thr84), and one Lys (Lys106) [11]. Sequence-based and structure-based sequence alignment results of the ComA protein in PDB showed >20% homology with a larger CheY family of regulatory domains. Members of this family include DosR [55], NarL [4], FixJ [7, 16], and CheY [10]. The activation mechanism in this family of regulatory domains has been studied extensively through comparisons of available native and phosphorylation-activated regulatory domain structures. According to DosR structure [55], sequence alignment results showed that K106 was located in the β5–α5 loop. I125 and T141 were located in the loop linker region away from the N-terminal domain in our predicted ComA structure. These data implicated that phosphorylation causes a movement of the Lys106 residue in ComA and allows conformational changes of surrounding residues, such as I125 and T141. According to the CheY family's general phosphorylation activation mechanism and the fact that these conserved residues Asp55, Thr 84, and Lys 106 were found in ComA, we propose that ComA may have a similar phosphorylation activation process to the CheY family partly. The unphosphorylated ComA protein in an inactive/closed conformation is in equilibrium with the opened/facilitated to be recognized conformation in solution. At a range of pH, ComA protein adopts multiple conformations, including one allowing Asp55 to be solvent-exposed part of the time. Phosphorylation of Asp55 is likely to promote a cascade of events that may be as follows. Phosphorylation of this critical Asp residue causes a movement of a conserved Lys (K106 in the ComA protein) in the β5–α5 loop to form a salt-bridge to the phosphoryl group. Phosphorylation also causes a conformational change of a conserved Thr residue (Thr 84 in the ComA protein) in strand β4 to form a hydrogen bond with a phosphoryl oxygen atom. This allows the side chain of the Ile and/or Thr residue in β5 to reorient from a solvent-exposed outward position to fill a pocket vacated by the Thr residue in a buried inward position, with concomitant conformational changes of surrounding residues.

The first strong evidence to support this hypothesis is that the mutation of Lys106 causes the inactivation of ComA protein. Secondly, in the native apo-CheY protein, the other areas are somehow keeping both the active site and the helix α4 in unstable conformations [11]. This means that CheY is an unstable protein. Moreover, the simulated 3D structure shows that the β4–α4 zone and the canonical (βα)5 fold are contained in ComA. These clues suggest that ComA is also an unstable protein and fitted to

basic structural need to a general phosphorylation activation mechanism. Lastly, the function of the two residues I125 and T141 located in the loop linker region are unknown because of few reports. Maria Solà *et al.* [42] introduced several mutations to the helix by following two strategies: increasing the intrinsic helical stability of helix α4, and improving the packing against the protein core. These mutants designed at helix α4 gained stability. These results show that decreasing the total negative charge of the active site, either by mutations that eliminate a negative polar residue or by the introduction of a positive charge such as the magnesium cation, increases the level of protein stability [42]. The report implicates that the intrinsic helical stability play a key role in the activity of ComA protein. In our work, two residues have been mutated to substitution, Ile for Thr and Thr for Pro. The possible cause for the reduction of activity is that two residues located in helix α5 substitution affected the stability of ComA because of the Pro residue side-chain's rigidity.

Different response regulators have different magnitudes of conformational change [15], and the outcome is different from case to case. Examples of phosphorylation-induced conformational changes include domain separation in the case of NarL [4], dimerization in the case of FixJ [7], and oligomerization and the ability to bind to different protein interaction partners with different binding affinities in the case of CheY [54]. Therefore, although there are only small differences in their global architectures, two-component response regulators act with great diversity at the functional level. Our study suggested that K106 may have a general function within this protein family, whereas I125 and T141 may be specific to ComA. Owing to the inclusion of I125 and T141 as important phosphorylated residues in ComA, the conformational change induced by phosphorylation is unique to ComA.

Protein structure and function analysis data demonstrated that the mutation of a few key amino acids greatly affected the biological activity of ComA. Our study indicated that the ComA protein has several key N-terminal amino acid residues that are likely to be involved in phosphorylated activation and induction to a cascade of conformational changes. Although future investigation is still needed to elucidate how these amino acids facilitate protein phosphorylation, combination, and transcription *in vivo* and *in vitro*, it is clear that the mutation of three key amino acids in ComA greatly affected the biological activity of *Bacillus subtilis*, such as surfactin production, spore formation, and antifungal activity.

Acknowledgments

We thank BGSC for the pMUTIN4 and pDG1728 plasmids. We thank Fang Wang, Ph.D, for supplying the surfactin for biochemical tests. This work was supported by the National Natural Science Foundation of China (30971950), China Postdoctoral Science Foundation (20080431108), and Jiangsu Postdoctoral Science Foundation (0801036B).

References

1. Ames, S. K., N. Frankema, and L. J. Kenney. 1999. C-Terminal DNA binding stimulates N-terminal phosphorylation of the outer membrane protein regulator OmpR from *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **96:** 11792–11797.
2. Ansaldi, M., D. Marolt, T. Stebe, I. M. Mulec, and D. Dubnau. 2002. Specific activation of the *Bacillus* quorum-sensing systems by isoprenylated pheromone variants. *Mol. Microbiol.* **44:** 1561–1573.
3. Arima, K., A. Kakinuma, and G. Tamura. 1968. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: Isolation, characterization and its inhibition of fibrin clot formation. *Biochem. Biophys. Res. Commun.* **31:** 488–494.
4. Baikalov, I., I. Schroder, M. Kaczor-Grzeskowiak, K. Grzeskowiak, R. P. Gunsalus, and R. E. Dickerson. 1996. Structure of the *Escherichia coli* response regulator NarL. *Biochemistry* **35:** 11053–11061.
5. Berka, R. M., J. Hahn, M. Albano, I. Draskovic, M. Persuh, X. Cui, A. Sloma, and W. Widner, D. Dubnau. 2002. Microarray analysis of the *Bacillus subtilis* K-state: Genome-wide expression changes dependent on ComK. *Mol. Microbiol.* **43:** 1331–1345.
6. Besson, F., F. Peypoux, G. Michel, and L. 1978. Identification of antibiotics of iturin group in various strains of *Bacillus subtilis*. *J. Antibiot. (Tokyo)* **31:** 284–288.
7. Birck, C., L. Mourey, P. Gouet, B. Fabry, J. Schumacher, P. Rousseau, D. Kahn, and J. P. Samama. 1999. Conformational changes induced by phosphorylation of the FixJ receiver domain. *Structure* **7:** 1505–1515.
8. Bourret, R. B., J. F. Hess, K. A. Borkovich, A. A. Pakula, and M. I. Simon. 1989. Protein phosphorylation in chemotaxis and two-component regulatory systems of bacteria. *J. Biol. Chem.* **264:** 7085–7088.
9. Chen, Z. and Z. Xu. 2000. Evaluation and utilization of antagonistic bacteria against rice sheath blight. *Chinese J. Rice Sci.* **14:** 98–102.
10. Cho, H. S., S. Y. Lee, D. Yan, X. Pan, J. S. Parkinson, S. Kustu, D. E. Wemmer, and J. G. Pelton. 2000. NMR structure of activated CheY. *J. Mol. Biol.* **297:** 543–551.
11. Cho, H. S., J. G. Pelton, D. Yan, S. Kustu, and D. E. Wemmer. 2001. Phosphoaspartates in bacterial signal transduction. *Curr. Opin. Struc. Biol.* **11:** 679–684.
12. Comella, N. and A. D. Grossman. 2005. Conservation of genes and processes controlled by the quorum response in bacteria: Characterization of genes controlled by the quorum-sensing transcription factor ComA in *Bacillus subtilis*. *Mol. Microbiol.* **57:** 1159–1174.
13. Core, L. and M. Perego. 2003. TPR-mediated interaction of RapC with ComA inhibits response regulator-DNA binding for competence development in *Bacillus subtilis*. *Mol. Microbiol.* **49:** 1509–1522.
14. Cutting, S. M. and C. R. Harwood. 1990. *Molecular Biological Methods for Bacillus*. John Wiley and Sons, Sussex, England.

15. Gardino, A. K., B. F. Volkman, H. S. Cho, S. Y. Lee, D. E. Wemmer, and D. Kern. 2003. The NMR solution structure of BeF3-activated Spo0F reveals the conformational switch in a phosphorelay system. *J. Mol. Biol.* **331:** 245–254.
16. Gouet, P., B. Fabry, V. Guillet, C. Birck, L. Mourey, D. Kahn, and J. P. Samama. 1999. Structural transitions in the FixJ receiver domain. *Structure* **7:** 1517–1526.
17. Grossman, A. D. 1995. Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*. *Annu. Rev. Genet.* **29:** 477–508.
18. Guerout-Fleury, A.-M., N. Frandsen, and P. Stragier. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. *Gene* **180:** 57–61.
19. Guillen, N., Y. Weinrauch, and D. A. Dubnau. 1989. Cloning and characterization of the regulatory *Bacillus subtilis* competence genes *comA* and *comB*. *J. Bacteriol.* **171:** 5354–5361.
20. Hamoen, L. W., H. Eshuis, J. Jongbloed, G. Venema, and D. van Sinderen. 1995. A small gene, designated *comS*, located within the coding region of the fourth amino acid activation domain of *srfA*, is required for competence development in *Bacillus subtilis*. *Mol. Microbiol.* **15:** 55–64.
21. Hosono, K. and H. Suzuki. 1983. Acylpeptides, the inhibitors of cyclic adenosine 3',5'-monophosphate phosphodiesterase. I. Purification, physicochemical properties and structures of fatty acid residues. *J. Antibiot. (Tokyo)* **36:** 667–673.
22. Jarmer, H., T. S. Larsen, A. Krogh, H. H. Saxild, S. Brunak, and S. Knudsen. 2001. Sigma A recognition sites in the *Bacillus subtilis* genome. *Microbiology* **147:** 2417–2424.
23. Jiang, M., R. Grau, and M. Perego. 2000. Differential processing of propeptide inhibitors of Rap phosphatases in *Bacillus subtilis*. *J. Bacteriol.* **182:** 303–310.
24. Kakinuma, A., M. Hori, M. Isono, G. Tamura and K. Arima. 1969. Determination of amino acid sequence in surfactin, a crystalline peptide lipid surfactant produced by *Bacillus subtilis*. *Agric. Biol. Chem.* **33:** 971–972.
25. Kleerebezem, M., L. E. Quadri, O. P. Kuipers, and V. W. de Vos. 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol. Microbiol.* **24:** 895–904.
26. Levitt, M. 1992. Accurate modeling of protein conformation by automatic segment matching. *J. Mol. Biol.* **226:** 507–533.
27. Levitt, M., M. Hirshberg, R. Sharon, and V. Daggett. 1995. Potential energy function and parameters for simulations of the molecular dynamics of proteins and nucleic acids in solution. *Comput. Phys. Commun.* **91:** 215–231.
28. Li, D., F. Nie, L. Wei, B. Wei, and Z. Chen. 2007. Screening of high-yielding biocontrol bacterium Bs-916 mutant by ion implantation. *Appl. Microbiol. Biol.* **75:** 1401–1408.
29. Magnuson, R., J. Solomon, and A. D. Grossman. 1994. Biochemical and genetic characterization of a competence pheromone from *B. subtilis*. *Cell* **77:** 207–216.
30. Msadek, T., F. Kunst, A. Klier, and G. Rapoport. 1991. DegS–DegU and ComP–ComA modulator–effector pairs control expression of the *Bacillus subtilis* pleiotropic regulatory gene *degQ*. *J. Bacteriol.* **173:** 2366–2377.
31. Mueller, J. P., G. Bukusoglu, and A. L. Sonenshein. 1992. Transcriptional regulation of *Bacillus subtilis* glucose starvation-inducible genes: Control of *gsiA* by the ComP–ComA signal transduction system. *J. Bacteriol.* **174:** 4361–4373.
32. Nakano, M. M., R. Magnuson, A. Myers, J. Curry, A. D. Grossman, and P. Zuber. 1991. *srfA* is an operon required for surfactin production, competence development, and efficient sporulation in *Bacillus subtilis*. *J. Bacteriol.* **173:** 1770–1778.
33. Nakano, M. M., M. A. Marahiel, and P. Zuber. 1988. Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *J. Bacteriol.* **170:** 5662–5668.
34. Nakano, M. M., L. A. Xia, and P. Zuber. 1991. Transcription initiation region of the *srfA* operon, which is controlled by the *comP–comA* signal transduction system in *Bacillus subtilis*. *J. Bacteriol.* **173:** 5487–5493.
35. Nakano, M. M. and P. Zuber. 1993. Mutational analysis of the regulatory region of the *srfA* operon in *Bacillus subtilis*. *J. Bacteriol.* **175:** 3188–3191.
36. Ogura, M., H. Yamaguchi, K. Kobayashi, N. Ogasawara, Y. Fujita, and T. Tanaka. 2002. Whole-genome analysis of genes regulated by the *Bacillus subtilis* competence transcription factor ComK. *J. Bacteriol.* **184:** 2344–2351.
37. Ogura, M., H. Yamaguchi, K. Yoshida, Y. Fujita, and T. Tanaka. 2001. DNA microarray analysis of *Bacillus subtilis* DegU, ComA and PhoP regulons: An approach to comprehensive analysis of *B. subtilis* two-component regulatory systems. *Nucl. Acids Res.* **29:** 3804–3813.
38. Roggiani, M. and D. Dubnau. 1993. ComA, a phosphorylated response regulator protein of *Bacillus subtilis*, binds to the promoter region of *srfA*. *J. Bacteriol.* **175:** 3182–3187.
39. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory Press, New York.
40. Schneider, K. B., T. M. Palmer, and A. D. Grossman. 2002. Characterization of *comQ* and *comX*, two genes required for production of ComX pheromone in *Bacillus subtilis*. *J. Bacteriol.* **184:** 410–419.
41. Simonovic, M. and K. Volz. 2001. A distinct meta-active conformation in the 1.1^{-A} resolution structure of wild-type ApoCheY. *J. Biol. Chem.* **276:** 28637–28640.
42. Solá, M., E. López-Hernández, P. Cronet, E. Lacroix, L. Serrano, M. Coll, and A. Párraga. 2000. Towards understanding a molecular switch mechanism: Thermodynamic and crystallographic studies of the signal transduction protein CheY. *J. Mol. Biol.* **303:** 213–225.
43. Solomon, J. M., B. A. Lazazzera, and A. D. Grossman. 1996. Purification and characterization of an extracellular peptide factor that affects two different developmental pathways in *Bacillus subtilis*. *Genes Dev.* **10:** 2014–2024.
44. Souza, C. D., M. M. Nakano, and P. Zuber. 1994. Identification of *comS*, a gene of the *srfA* operon that regulates the establishment of genetic competence in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 9397–9401.
45. Tortosa, P., L. Logsdon, B. Kraigher, Y. Itoh, I. Mandic-Mulec, and D. Dubnau. 2001. Specificity and genetic polymorphism of the *Bacillus* competence quorum-sensing system. *J. Bacteriol.* **183:** 451–460.
46. Tortosa, P. and D. Dubnau. 1999. Competence for transformation: A matter of taste. *Curr. Opin. Microbiol.* **2:** 588–592.
47. Turgay, K., J. Hahn, J. Burghoorn, and D. Dubnau. 1998. Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *EMBO J.* **17:** 6730–6738.

48. Vagner, V., E. Dervyn, and S. D. Ehrlich. 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144:** 3097–3104.
49. van Sinderen, D., S. Withoff, H. Boels, and G. 1990. Isolation and characterization of comL, a transcription unit involved in competence development of *Bacillus subtilis*. *Mol. Gen. Genet.* **224:** 396–404.
50. Vanittanakom, N., W. Loeffler, and U. J. G. Koch. 1986. Fengycin - a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. *J. Antibiot. (Tokyo)* **39:** 888–901.
51. Volkman, B. F., D. Lipson, D. E. Wemmer, and D. Kern. 2001. Two-state allosteric behavior in a single-domain signaling protein. *Science* **291:** 2429–2433.
52. Weinrauch, Y., N. Guillen, and D. A. Dubnau. 1989. Sequence and transcription mapping of *Bacillus subtilis* competence genes *comB* and *comA*, one of which is related to a family of bacterial regulatory determinants. *J. Bacteriol.* **171:** 5362–5375.
53. Weinrauch, Y., R. Penchev, E. Dubnau, I. Smith, and D. Dubnau. 1990. A *Bacillus subtilis* regulatory gene product for genetic competence and sporulation resembles sensor protein members of the bacterial two-component signal-transduction systems. *Genes Dev.* **4:** 860–872.
54. Welch, M., K. Oosawa, S. Aizawa, and M. Eisenbach. 1993. Phosphorylation-dependent binding of a signal molecule to the flagellar switch of bacteria. *Proc. Natl. Acad Sci. U.S.A.* **90:** 8787–8791.
55. Wisedchaisri, G., M. Wu, D. R. Sherman, and W. G. Hol. 2008. Crystal structures of the response regulator DosR from *Mycobacterium tuberculosis* suggest a helix rearrangement mechanism for phosphorylation activation. *J. Mol. Biol.* **378:** 227–242.