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Novel Methods for Genetic Transformation of Natural *Bacillus subtilis* Isolates Used To Study the Regulation of the Mycosubtilin and Surfactin Synthetases▽†

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Natural isolates of *Bacillus subtilis* are often difficult to transform due to their low genetic competence levels. Here we describe two methods that stimulate natural transformation. The first method uses plasmid pGSP12, which expresses the competence transcription factor ComK and stimulates competence development about 100-fold. The second method stimulates Campbell-type recombination of DNA ligation mixtures in *B. subtilis* by the addition of polyethylene glycol. We employed these novel methods to study the regulation of the synthetases for the lipopeptide antibiotics mycosubtilin (*myc*) and surfactin (*srfA*) in *B. subtilis* strain ATCC 6633. By means of *lacZ* reporter fusions, it was shown that the expression of *srfA* is >100 times lower in strain ATCC 6633 than in the laboratory strain *B. subtilis* 168. Expression of the *myc* operon was highest in rich medium, whereas *srfA* expression reached maximal levels in minimal medium. Further genetic analyses showed that the *srfA* operon is mainly regulated by the response regulator ComA, while the *myc* operon is primarily regulated by the transition-state regulator AbrB. Although there is in vitro evidence for a synergistic activity of mycosubtilin and surfactin, the expression of both lipopeptide antibiotics is clearly not coordinated.

The endospore-forming soil bacterium *Bacillus subtilis* is able to produce more than two dozen antibiotics with an amazing variety of structures. Most of these components show antimicrobial or antiviral activity (44). *B. subtilis* is amenable towards genetic manipulations, thanks to its ability to become naturally genetically competent. This feature would facilitate study into the production and engineering of these antibiotics were it not that the highly competent laboratory strains have lost the capacity to produce almost all antibiotics. Unfortunately, natural *B. subtilis* isolates that do make antibiotics appear to be difficult to transform, due to a much reduced (natural) level of competence. In this study, we describe two methods to facilitate the genetic transformability of *B. subtilis* strains and show their value by studying the regulation of the mycosubtilin (*myc*) and surfactin (*srfA*) synthetase operons in *B. subtilis* ATCC 6633.

Many bacteria produce small, modified peptides that are synthesized nonribosomally by large multienzyme complexes, i.e., the peptide synthetases (40). Owing to important medical properties of several of these peptides and to the promising engineering prospects of the peptide synthetases (11), there is a growing interest in these multienzyme complexes. Most nonribosomally synthesized peptides produced by *B. subtilis* are cyclic peptides with a fatty acid modification, such as surfactin, fengycin, and the members of the iturin family, including mycosubtilin. *B. subtilis* strain ATCC 6633 produces two lipopeptides, surfactin and mycosubtilin (8). Surfactin consists of a cyclic heptapeptide closed into a lactone ring by a β-hydroxy fatty acid. This lipopeptide exhibits strong antiviral and hemolytic activities but only a limited antibacterial activity. Surfactin seems to also be required for gliding motility (20, 30). Mycosubtilin consists of a cyclic heptapeptide closed into an amide ring by a β-amino fatty acid. Mycosubtilin exhibits a strong antifungal activity, especially against filamentous fungi (23).

In contrast to the structure and function of peptide synthetases, not much is known about the regulation of expression of these large enzyme complexes. Only the transcriptional regulation of the surfactin synthetase operon has been studied extensively, because of its role in the development of genetic competence (6, 14). Expression of *srfA* is medium and growth phase dependent and increases sharply at the transition from exponential to stationary-phase growth. In addition, surfactin production is associated with increased cell densities. Expression of *srfA* is mainly governed by the two-component regulatory system ComA and ComP (29, 35). Phosphorylation of ComA stimulates binding of this response regulator to the promoter of *srfA*, which induces the expression of this operon. The membrane kinase ComP senses the accumulation of ComX pheromone in the medium and activates ComA (33). Binding of ComA to the *srfA* promoter is inhibited by RapC. Like that of most members of the Rap family, the activity of

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TABLE 1. Strains and plasmids

B. subtilis strain or plasmid	Relevant genotype/characteristics	Source or reference
Strains		
168-7G5	Derivative of *B. subtilis* 168; surfactin positive	46
168-8G5	Derivative of *B. subtilis* 168; surfactin negative	2
AG665	Cm^r Δ*spo0H*	19
ATCC6633	Mycosubtilin positive; surfactin positive	9
BD1777	Cm^r Δ*comA*	12
BV12E12	(ATCC 6633) Km^r *mycA-lacZ*; mycosubtilin negative	This work
BV12E13	(ATCC 6633) Km^r *srfAD-lacZ*	This work
BV12E14	(ATCC 6633) Km^r *myc-lacZ*; mycosubtilin positive	This work
BV12E15	(168-8G5) Km^r *srfAD-lacZ*; surfactin negative	This work
BV12E16	(ATCC 6633) Cm^r Km^r *mycA-lacZ* Δ*comA*	This work
BV12E18	(ATCC 6633) Cm^r Km^r *mycA-lacZ* Δ*abrB*	This work
BV12E20	(ATCC 6633) Cm^r Km^r *mycA-lacZ* Δ*sinR*	This work
BV12E22	(ATCC 6633) Sp^r Km^r *mycA-lacZ* Δ*cssS*	This work
BV12E24	(ATCC 6633) Cm^r Km^r *mycA-lacZ* Δ*degU*	This work
BV12E25	(ATCC 6633) Cm^r Km^r *mycA-lacZ* Δ*srfAA*	This work
BV12E27	(168-8G5) Km^r *mycp-lacZ*; surfactin negative	This work
BV12E28	(168-7G5) Km^r *mycp-lacZ*; surfactin positive	This work
BV12E29	(ATCC 6633) Cm^r Km^r *mycA-lacZ* Δ*spo0K*	This work
BV12E31	(ATCC 6633) Cm^r Km^r *mycA-lacZ* Δ*spo0H*	This work
BV12E32	(ATCC 6633) Cm^r Km^r *srfAD-lacZ* Δ*spo0K*	This work
BV12E33	(ATCC 6633) Cm^r Km^r *srfAD-lacZ* Δ*spo0H*	This work
BV12E35	(168-8G5) Km^r *mycp-lacZ* Δ*comA*; surfactin negative	This work
BV12E39	(168-8G5) Km^r *mycp-lacZ* Δ*abrB*; surfactin negative	This work
BV12E40	(168-7G5) Km^r *srfAD-lacZ*; surfactin positive	This work
BV12I11	(ATCC 6633) Cm^r Km^r *srfAD-lacZ* Δ*comA*	This work
BV12I37	(ATCC 6633) Cm^r Km^r *mycA-lacZ* Δ*codY*	This work
BV12I38	(ATCC 6633) Cm^r Km^r *srfAD-lacZ* Δ*codY*	This work
BV15D29	Sp^r Km^r Δ*cssS*	18
IS432	Cm^r Δ*sinR*	10
JH12586	Cm^r Δ*abrB*	31
KI566	Cm^r Δ*Spo0K*	37
Plasmids		
pGSP12	Em^r; contains *comK*	47
pLGW300	Km^r; contains promoterless *spo0V-lacZ* fusion	49
pUC19C	Amp^r Cm^r	Lab collection, unpublished

RapC is dependent on the accumulation of a specific secreted pentapeptide, PhrC in this case. PhrC is taken up via oligopeptide permeases and represses the activity of RapC (4, 42). In addition, the *srfA* promoter is under direct negative control of the transcription factor CodY (39). Several other transcription factors, such as DegU and PerR, influence *srfA* expression as well, and it is evident that regulation of this antibiotic is part of a complex cascade that governs multiple differentiation pathways in *B. subtilis* (13, 16, 17).

Biochemical experiments have shown that surfactin displays a synergistic effect on the biological properties of iturin A (25). Mycosubtilin belongs to the iturin family, and *B. subtilis* strain ATCC 6633 produces both mycosubtilin and surfactin (8). Therefore, we wondered whether the production of these antibiotics is coordinated in this strain. Since ATCC 6633 develops poor levels of genetic competence, methods were developed to facilitate natural transformation. The first method makes use of a plasmid that increases the concentration of the competence transcription factor ComK. The second method is based on a ligation procedure that facilitates Campbell-type recombination in *B. subtilis*.

MATERIALS AND METHODS

General methods and materials. Bacterial strains and plasmids used in this study are listed in Table 1. Molecular cloning and PCR procedures were carried out using standard techniques. Plasmids constructed by PCR were verified by sequencing. Oligonucleotides used for PCR are listed in Table S1 in the supplemental material. *B. subtilis* sporulation and minimal media were prepared as described by Schaeffer et al. (38) and Spizizen (43), respectively, and TY broth was used as rich medium. *B. subtilis* chromosomal DNA was purified according to the method of Venema et al. (50). Reverse transcription-PCR (RT-PCR) was performed using Superscript reverse transcriptase (Roche Diagnostics) and primers DF1, DF2, and FF2 (see Table S1 in the supplemental material). Total RNA isolations for RT-PCR were performed using a High Pure RNA isolation kit (Roche Diagnostics).

Transformations. Transformation protocols for competent *B. subtilis* cells were based on those of Spizizen, with some adjustments (15, 43). Protoplast transformation was performed as described by Chang and Cohen (3). Transformation of pGSP12-containing *B. subtilis* strains was done as follows. An overnight culture was grown in minimal medium with 2.5 μg/ml erythromycin at 37°C, with continuous shaking at 300 rpm. After 100-fold dilution of the overnight culture in minimal medium, incubation was continued, and the optical density at 600 nm (OD_{600}) was monitored. Two hours after the transition from exponential to stationary-phase growth, 1 μg of DNA was added to 0.5 ml of competent cells. Samples were kept at 37°C with shaking. After 20 min, 0.3 ml of TY medium was added, and growth was continued for another 30 min, after which the cells were plated on selective TY-agar plates.

PEG ligation. All ligation reactions were performed overnight at room temperature, using T4 ligase and buffer from Roche Diagnostics in a total volume of 30 μl. For polyethylene glycol (PEG) ligations, a PEG 8000 solution (heat sterilized) was added to a final concentration of 15% (32).

Reporter gene fusions. A detailed description of the construction of the different reporter gene fusions can be found in the supplemental material. To measure the expression and regulation of the *srfA* and *myc* operons, *lacZ* reporter gene fusions were made. For the construction of the transcriptional *myc-lacZ* fusion in *B. subtilis* ATCC 6633 (strain BV12E12), an internal part of *mycA*, obtained by PCR, was cloned into pLGW300. This plasmid contains the ribosomal binding site of the *B. subtilis spoVG* gene fused to a promoterless *lacZ* gene (49). In order to determine the possible effects of mycosubtilin production on the expression of *myc*, a transcriptional *lacZ* fusion was also made downstream of *myc*, without disrupting the operon (strain BV12E14).

To study the expression and transcriptional regulation of *myc* in *B. subtilis* 168, we inserted a transcriptional fusion of the *myc* promoter region with *lacZ* into the genome of the *B. subtilis* 168 derivative strain 8G5 (denoted 168-8G5), resulting in strain BV12E27. The *myc* promoter-*lacZ* fusion was inserted between *dacC* and *ppsA*, identical to the position occupied by *myc* in *B. subtilis* ATCC 6633 (8). Because plasmids containing the mycosubtilin promoter are not stable in *Escherichia coli*, this organism could not be used as the cloning host, and the ligation products were transformed directly into competent 168-8G5 cells. To increase the efficiency of this process, ligation was performed in the presence of PEG, as described above.

To examine whether the expression and transcriptional regulation of *srfA* in *B. subtilis* ATCC 6633 are comparable to those in *B. subtilis* 168-8G5, transcriptional fusions of *srfA* with *lacZ* were made in both strains, resulting in strains BV12E13 and BV12E15, respectively. *B. subtilis* 168 does not produce surfactin due to a defective phosphopantetheinyl transferase encoded by *sfp* (28). To test whether surfactin production influences *srfA* expression, the *srfA-lacZ* reporter fusion was also introduced into strain 168-7G5, which has an intact *sfp* gene and produces surfactin (5). Surfactin production of the resulting strain, BV12E40, was confirmed using blood agar plates, as described below.

Mutational analyses. To determine the possible involvement of the AbrB, CodY, ComA, CssS, DegU, SinR, and Spo0K proteins in the transcriptional regulation of *srfA* and *myc* in *B. subtilis* ATCC 6633, the genes encoding these proteins were mutated. This was done mostly by transformation with chromosomal DNAs of *B. subtilis* strains already harboring the desired mutations, marked with an antibiotic resistance gene. The resulting strains are listed in Table 1, and a detailed description of the construction of these strains can be found in the supplemental material.

β-Galactosidase assays. β-Galactosidase activities were used to measure the expression levels of the lipopeptide synthetase operons in strains harboring the *lacZ* fusions. For this purpose, samples were taken at hourly intervals for OD_{600} readings and β-galactosidase assays (49). β-Galactosidase activities were expressed as activity units per OD_{600} unit.

Bioassays for lipopeptide production. The production of the lipopeptides surfactin and mycosubtilin was measured using bioassays described by Besson et al. and Mulligan and Gibbs, respectively (1, 27). For mycosubtilin production, *Saccharomyces cerevisiae* G910 was used as an indicator, and for surfactin production, sheep blood was used as an indicator.

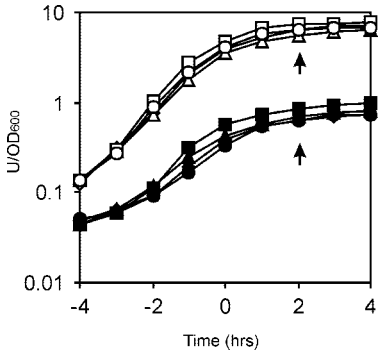
RESULTS

Stimulating transformation by a plasmid-located copy of *comK*. The genes coding for the DNA uptake and integration machinery are activated by a single transcription factor, the competence transcription factor ComK. Competence is a complicated process of physiological differentiation in which cell division is blocked as well. It is therefore not surprising that ComK expression is tightly regulated (reviewed in reference 16). In fact, the *comK* promoter is directly controlled by no fewer than five different transcription regulators, namely, ComK, Rok, CodY, AbrB, and DegU. ComK stimulates its own expression with the help of the response regulator DegU. Rok, CodY, and AbrB are repressors of the *comK* promoter. In addition, there is a specific adaptor protein, MecA, that targets ComK for degradation by the ClpCP system. This proteolytic control mechanism is regulated by the quorum-sensing pathways, in which, surprisingly, *srfA* also takes part. Even when conditions are optimal, only about 10 to 20% of cells in a *B. subtilis* 168 culture induce ComK. The mechanism of this bistable expression was recently solved (24, 41). The *comK* promoter has a low basal level of expression that fluctuates stochastically between individual cells. When in certain cells the concentration of ComK reaches a threshold level, the autostimulatory induction of *comK* starts to kick in, and only these cells will fully activate *comK* and become competent. All of the regulatory proteins listed above, aside from ComK itself, are there to keep the threshold level high to prevent premature activation of autostimulatory *comK* expression. The low levels of competence attained in most natural isolates of *B. subtilis* are likely due to ComK threshold levels that are kept too high in most cells to initiate *comK* autostimulation. Of course, it is also possible that the *comK* gene is mutated in some strains.

Several years ago, van Sinderen and Venema (48) showed that the introduction of a low-copy-number plasmid containing the *comK* gene (pGSP12) stimulates competence development and bypasses the normal medium requirements, resulting in competence development in rich medium. This observation can now be explained by assuming that the expression of the additional copies of *comK* lowers the *comK* activation threshold level substantially. We reasoned that plasmid pGSP12 could be used to stimulate genetic competence in natural isolates of *B. subtilis*. To test this, we introduced pGSP12 into *B. subtilis* strains ATCC 6633 and ATCC 21332 by means of conventional protoplast transformation. Competence was measured by transforming the different strains with chromosomal DNA from a *B. subtilis* strain containing a chloramphenicol resistance marker. The results are shown in Fig. 1. In minimal (competence) medium, the presence of pGSP12 increased transformation efficiencies almost 100-fold, and even in rich TY broth, which normally inhibits competence development, a substantial number of chloramphenicol-resistant transformants were obtained when pGSP12 was present.

***srfA* expression is low in *B. subtilis* ATCC 6633.** Once we had raised the competence of ATCC 6633 to convenient levels, it was possible to use classic *B. subtilis* genetic strategies to examine gene regulation in this strain (all ATCC 6633 derivatives were made using pGSP12, which is not mentioned further in the text). We first examined the expression of *srfA*. In *B. subtilis* 168, expression of *srfA* is induced at the end of the exponential growth phase and reaches the highest levels in minimal medium (49). To examine whether *srfA* shows similar medium- and growth phase-dependent expression in *B. subtilis* ATCC 6633, a *srfA-lacZ* transcriptional reporter gene fusion was constructed (BV12E13). As a control, we used *B. subtilis* strain 8G5, which is a derivative of strain 168 (BV12E15) (2). For clarity, we refer to this strain as *B. subtilis* 168-8G5. The activity of the promoter of *srfA* was measured in rich, minimal, and sporulation media at hourly intervals. Figure 2 shows that the expression of *srfA* in ATCC 6633 was about 200-fold lower than that in strain 168-8G5 (note the different scales). Despite this much lower expression level, the growth phase-related induction and the medium dependency of *srfA* expression were comparable in both strains.

***srfA* induction is ComA but not CodY dependent in *B. subtilis* ATCC 6633.** To assess whether regulation of *srfA* in *B. subtilis* ATCC 6633 is governed by the same regulators as that



Number of transformants per OD600:

	ATCC6633	
	minimal medium	rich medium
- pGSP12	0 - 2	0
+ pGSP12	444 - 486	55 - 87

	ATCC21332	
	minimal medium	rich medium
- pGSP12	13 - 19	0 - 2
+ pGSP12	700 - 762	70 – 96

FIG. 1. pGSP12-stimulated transformation of *B. subtilis* ATCC 6633 and ATCC 21332. Growth curves for *B. subtilis* ATCC 6633, with (▲/△) and without pGSP12 (■/□), and *B. subtilis* ATCC 21332, with (●/○) and without (◆/◇) pGSP12, are presented in the graph. Closed symbols refer to growth in minimal medium, and open symbols refer to growth in rich medium. The time scale indicates hours before and after the transition from the exponential to the stationary growth phase, and arrows indicate the time of transformation. The numbers of transformants obtained are shown in the table.

in *B. subtilis* 168, we introduced several mutations into our *srfA-lacZ* reporter strain. The β-galactosidase activities of the different mutants were measured in minimal medium, and the results are depicted in Fig. 2C. First, the gene encoding the main activator of *srfA* in *B. subtilis* 168, *comA*, was deleted (BV12I11). From the resulting β-galactosidase levels, it is clear that also in the ATCC 6633 background, ComA is essential for *srfA* expression. The transcription factor CodY is a known repressor of *srfA*. However, in strain ATCC 6633, a *codY* mutation had almost no effect on *srfA* expression (BV12I38). We also introduced mutations into the σ^H-encoding gene *spo0H* and the oligopeptide permease gene *spo0K* (BV12E33 and BV12E32). Both genes modulate PhrC levels (22). Due to the stimulating effect of PhrC on ComA activity, it was not surprising that mutations in *spo0H* and *spo0K* also affected *srfA* expression in ATCC 6633. However, it should be mentioned that the negative effects of a *spo0K* mutant were not as dramatic as those published for *B. subtilis* 168 (13).

***myc* expression in *B. subtilis* ATCC 6633.** To study expression of the mycosubtilin synthetase operon, we constructed a *myc-lacZ* reporter gene fusion (BV12E12). Figure 3A shows the β-galactosidase activities of an ATCC 6633 strain containing this reporter fusion and grown in different media. The levels of *myc* expression were comparable to those found for *srfA* in ATCC 6633 and were also maximal in the stationary phase of growth. In contrast to the case for *srfA*, the expression of *myc* was highest in rich medium and lowest in minimal medium. Sporulation medium gave intermediate levels of expression.

The different responses to medium compositions suggested that there are differences in the regulation of *srfA* and *myc*. A *comA* mutant (BV12E16) confirmed this, as the introduction of a *comA* mutation did not lead to reduced β-galactosidase levels (result not shown). We tested several other regulators involved in post-exponential-phase gene expression (*abrB*, *codY*, *cssS* [18], *degU*, *sinR* [10], *spo0H*, and *spo0K*), but only an *abrB* mutation (BV12E18) gave a strong response, resulting in a fivefold increase in *myc* induction (Fig. 3B [note the difference in scale]). Of the other regulators tested, the *spo0H* mutant gave a mild reduction in β-galactosidase activity (BV12E31). These data clearly show that the surfactin and mycosubtilin synthetase operons are regulated differently in *B. subtilis* ATCC 6633.

Construction of a *myc* promoter fusion by PEG ligation. Since *srfA* expression was much stronger in 168-8G5 than in the ATCC 663 background, we were curious whether this would also be the case for the expression of *myc*. The difficulty

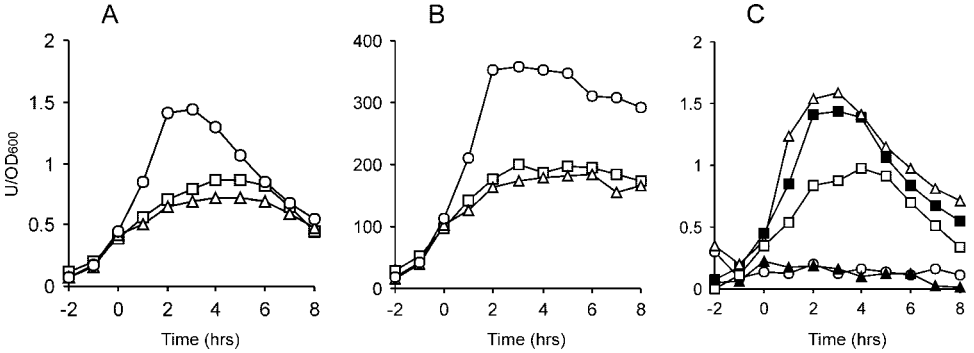


FIG. 2. *srfA-lacZ* expression in *B. subtilis*. The expression levels of *srfA-lacZ* in strains ATCC 6633 (A) and 168-8G5 (B), grown in rich medium (□), minimal medium (○), and sporulation medium (△), are depicted. (C) Effects of various mutations on the expression of *srfA-lacZ* in *B. subtilis* ATCC 6633. The following cultures were grown in minimal medium: ■, wild type; ○, Δ*comA* mutant; △, Δ*codY* mutant; ▲, Δ*spo0H* mutant; and □, Δ*spo0K* mutant. The time scales refer to hours before and after the transition from exponential to stationary-phase growth (defined as time zero). β-Galactosidase activities are presented in activity units per OD_{600} unit.

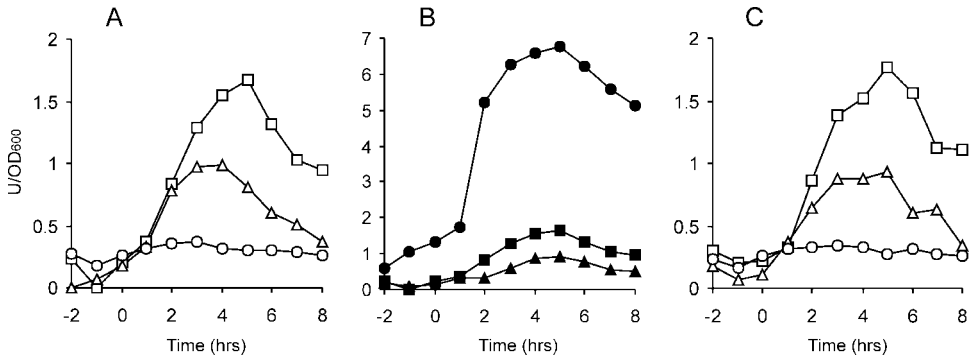


FIG. 3. *myc-lacZ* expression in *B. subtilis*. (A) Expression levels of *myc-lacZ* in strain ATCC 6633, grown in rich medium (□), minimal medium (○), and sporulation medium (△). (B) Effects of various mutations on the expression of *myc-lacZ* in *B. subtilis* ATCC 6633. The following cultures were grown in rich medium: ■, wild type; ●, Δ*abrB* mutant; and ▲, Δ*spo0H* mutant. (C) Expression profiles of a *myc-lacZ* reporter fusion in *B. subtilis* 168-8G5, grown in rich medium (□), minimal medium (○), and sporulation medium (△). The time scales refer to hours before and after the transition from exponential to stationary-phase growth (defined as time zero). β-Galactosidase activities are presented in activity units per OD_{600} unit.

is that *B. subtilis* 168 does not contain a *myc* operon. Instead, this strain contains, at the same locus, another lipopeptide synthetase operon, that for fengycin (8). Thus, the flanking regions of the *myc* operon in ATCC 6633 are exactly the same as the flanking regions of the fengycin synthetase operon in strain 168. From an evolutionary point of view, this exchange of antibiotic synthetases is fascinating, but for our purpose, the absence of *myc* sequences in 168 was a complicating factor. We decided to construct a *myc* promoter-*lacZ* fusion and to integrate this reporter fusion at the same locus as that in ATCC 6633, that is, downstream of *dacC*. The promoter of *myc* is likely to reside in the 960-bp intergenic region between *dacC* and *fenF*, with the latter being the first gene of the *myc* operon (8). This was confirmed by an RT-PCR that located a transcriptional start site within a 30-bp region 180 bp upstream of *fenF* (data not shown). Within this 30-bp region, there is a perfect −10 consensus sequence for a σ^A-dependent promoter, but no clear −35 consensus sequence is present. To be sure, the whole intergenic region between *dacC* and *fenF* was chosen for the *lacZ* reporter fusion. Unfortunately, cloning of the *myc* promoter into the cloning host *E. coli* appeared to be impossible. We therefore had to consider cloning the *myc* promoter-*lacZ* fusion directly into *B. subtilis* (see Materials and Methods for details). Campbell integrations in competent *B. subtilis* cells require the uptake of multimeric DNA (7). It is known that the presence of PEG during the ligation reaction inhibits the formation of closed circular monomeric DNA (32), thus resulting in a large percentage of large linear multimeric DNA molecules, the ideal substrate for Campbell-type integrations in *B. subtilis*. Moreover, macromolecular crowding caused by PEG strongly stimulates the ligation reaction itself. We found that the *B. subtilis* transformation efficiencies for DNA ligation products increased 30- to 60-fold when PEG 8000 was included in the ligation mixture (data not shown). This facilitated the direct integration of a *myc* promoter-*lacZ* fusion into the genome of *B. subtilis* 168-8G5 (BV12E27). As shown in Fig. 3C, the *myc* expression profiles for 168-8G5 were the same as those for ATCC 6633 (BV12E12). We also tested mutations in different regulators, with the same effect; also, in *B. subtilis* 168-8G5, the *myc* promoter was strongly induced, up to fivefold, when *abrB* was deleted (BV12E39), whereas a mutation in *comA* (BV12E35) had no effect (data not shown).

DISCUSSION

Here we describe two methods to facilitate genetic studies of *B. subtilis*. In the first method, we boost the expression of the competence transcription factor ComK by the introduction of plasmid pGSP12. There are several reasons why such an approach is of interest, including (i) pGSP12 can easily be artificially introduced into low-competence or noncompetent *B. subtilis* strains by means of protoplast transformation or electroporation; (ii) if necessary, the plasmid can be removed by plasmid curing; and (iii) the method will introduce a new *comK* gene in those strains that have lost an active copy of the gene. It is likely that the efficiency of homologous recombination depends on the measure of homology between DNA fragments. During the isolation and sequencing of the *myc* operon, we noticed sequence differences of up to 2% in certain genes compared with the sequence of *B. subtilis* 168. The differences in genome sequence between both strains might explain why we had difficulties deleting *codY* or *degU* from *B. subtilis* ATCC 6633 when using donor DNAs from strain 168 derivatives. Therefore, when chromosomal DNA from a different donor strain is used for transformation, good levels of competence are essential. Introduction of plasmid pGSP12 can help with this. The second method describes how ligation mixtures can be transformed into *B. subtilis* more efficiently when PEG is present in the reaction mix. In fact, the use of ligation products for Campbell-type integrations in *B. subtilis* renders the use of shuttle vectors redundant. We have used this method on several occasions to integrate antibiotic markers into the genome. Recently, a method for *B. subtilis* protoplast electroporation was also described, and it shows potential for transformation of natural *B. subtilis* isolates (36).

The two methods were applied to study the regulation of mycosubtilin synthesis. It appeared that the expression of the mycosubtilin synthetase is regulated differently from that of surfactin. We also tested whether the actual production of the lipopeptide antibiotics would trigger the induction of synthe-

tases, but neither expression of *srfA* nor that of *myc* showed any change upon the presence of surfactin and/or mycosubtilin in the medium (data not shown).

The response regulator ComA is one of the main regulators of *srfA* expression. The regulation of *srfA* has been studied extensively because this large operon harbors a small gene, *comS*, which is essential for competence development. ComS blocks the degradation of ComK by the ClpCP protease complex (45). The low expression level of *srfA*, and therefore *comS*, in ATCC 6633 might explain the low level of competence of this strain. We do not know why *srfA* expression is much lower in ATCC 6633 than in the 168 strain. In *B. subtilis* 168, a mutation of the oligopeptide permease Spo0K has a detrimental effect on *srfA* expression because the small secreted signaling peptide PhrC cannot be taken up anymore, and therefore inhibition of ComA by ParC is not blocked (42). The genome of *B. subtilis* harbors another oligopeptide permease operon, namely, *app*. In strain 168, this gene is mutated, and the encoded permease is inactive (21). Presumably, this alternative permease is active in strain ATCC 6633, which would explain why a *spo0K* mutation shows only a mild effect on expression of *srfA*. Why a mutation in the regulator *codY* had no consequences for *srfA* expression in *B. subtilis* ATCC 6633 is unknown.

The ComA/ComP signal transduction pathway is the main quorum-sensing system in *B. subtilis*. It is therefore not surprising that this control mechanism directs the synthesis of antibiotics. In fact, the synthesis of the antibiotics bacilysin in *B. subtilis* and lichenysin A in *Bacillus licheniformis* is also dependent on ComA (26, 51). Nevertheless, the expression of mycosubtilin is governed by another regulatory cascade, in which AbrB forms the center. AbrB is a very pleiotropic regulator and is one of the main transition-state regulators in *B. subtilis*. In the case of the tyrocidin operon of *Bacillus brevis*, there is also evidence for AbrB-dependent control (26). Since σ^H is indirectly involved in the repression of *abrB* during the transition to stationary-phase growth (34), this would explain the reduced expression of *myc* in a *spo0H* background. According to Fig. 3A, an *abrB* mutant of ATCC 6633 still shows growth-phase-dependent induction of *myc*, so apparently there are more regulators involved in the regulation of this operon. With the new genetic tools described in this paper, it will now be easier to examine this in more detail.

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