

A master regulator for biofilm formation by *Bacillus subtilis*

Daniel B. Kearns,¹ Frances Chu,¹ Steven S. Branda,² Roberto Kolter,² and Richard Losick^{1*}

¹Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA.

²Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA.

Summary

Wild strains of *Bacillus subtilis* are capable of forming architecturally complex communities of cells known as biofilms. Critical to biofilm formation is the *eps* operon, which is believed to be responsible for the biosynthesis of an exopolysaccharide that binds chains of cells together in bundles. We report that transcription of *eps* is under the negative regulation of SinR, a repressor that was found to bind to multiple sites in the regulatory region of the operon. Mutations in *sinR* bypassed the requirement in biofilm formation of two genes of unknown function, *y1bF* and *ymcA*, and *sinI*, which is known to encode an antagonist of SinR. We propose that these genes are members of a pathway that is responsible for counteracting SinR-mediated repression. We further propose that SinR is a master regulator that governs the transition between a planktonic state in which the bacteria swim as single cells in liquid or swarm in small groups over surfaces, and a sessile state in which the bacteria adhere to each other to form bundled chains and assemble into multicellular communities.

Introduction

Biofilms are architecturally complex communities of microorganisms in which the cells are held together by an extracellular matrix, typically containing exopolysaccharides (EPSs), proteins and even nucleic acids (Hall-Stoodley *et al.*, 2004). An attractive organism in which to identify, and investigate the function of, genes involved in biofilm formation is *Bacillus subtilis*. This Gram-positive, spore-forming, soil bacterium is highly accessible to

manipulation by the techniques of classical and molecular genetics, and wild (undomesticated) strains of *B. subtilis* form robust biofilms both at liquid/air interfaces and on solid surfaces (Branda *et al.*, 2001). In standing liquid medium, cells of *B. subtilis* switch from a submerged, highly motile planktonic state in which the bacteria swim as single cells, to a non-motile state in which the cells grow as bundled chains that rise to the surface and form a robust pellicle. On the surface of agar plates, the cells form colonies with elaborate architecture, including aerial structures that resemble fruiting bodies and that preferentially produce spores at their tips (Branda *et al.*, 2001). Pellicles and colonies share the common feature that growth in filamentous chains of cells is associated with, if not essential for, architectural complexity. Indeed, this capacity to grow in bundled chains was recognized by Ferdinand Cohn in his report on the discovery of *B. subtilis* (Cohn, 1872).

Previous work has revealed a large number of genes that govern biofilm formation in *B. subtilis* (Branda *et al.*, 2001; 2004; Hamon and Lazazzera, 2001; Hamon *et al.*, 2004). These include regulatory genes involved in the early stages of sporulation (*spo0A*, *spo0H* and *abrB*), genes that are putatively involved in EPS production (*yhxB* and the 15-gene-long *yveK-yvfF* operon, which is herein renamed *epsA-O'*), a gene encoding a putative phosphatase (*yqeK*), a gene involved in the production of the surfactant, surfactin (*sfp*), a gene encoding a signal peptidase (*sipW*), a gene encoding an ABC transporter subunit (*ecsB*), and two genes of largely unknown function whose inferred products exhibit substantial amino acid-sequence similarity to each other (*y1bF* and *ymcA*). Key challenges for the future are to comprehensively identify genes involved in biofilm formation, to determine which genes fall into common pathways, and to elucidate how these pathways operate.

¹We noticed that the predicted products of two adjacent genes (originally named *yvfA* and *yvfB*; Kunst *et al.*, 1997) showed sequence similarity to the N and C terminal regions respectively, of an orthologous group of proteins (Tatusov *et al.*, 1997) (COG2244, which includes *E. coli* WzxE and other proteins thought to mediate export of O-antigen polysaccharides; Liu, D. *et al.*, 1996; Rick *et al.*, 2003), suggesting the possibility that a single ORF had been annotated as two resulting from a sequencing error. Indeed, we found that *B. subtilis* 168 and 3610 contain a G-C insertion in codon 101 of the reported *yvfA* sequence, resulting in the merger of *yvfA* and *yvfB* into a single, 505-codon-long ORF, which we have named *epsK*.

Here we describe one such pathway that governs the switch from growth as motile cells to growth as biofilm-forming chains of cells. In addressing the issue of the role of cell chains in biofilm formation, our attention was drawn to the regulatory genes *sinR* and *sinI*. In commonly used laboratory strains, a *sinR* mutation causes the formation of rugose colonies in which cells grow constitutively as chains of non-motile cells (Fein, 1979; Gaur *et al.*, 1986; Sekiguchi *et al.*, 1988; 1990). Cells of a *sinI* mutant, in contrast, are always motile and do not form chains (Bai *et al.*, 1993). The *sinR* gene is known to encode a DNA-binding protein, and *sinI* is known to encode an antagonist of the *sinR* gene product (SinR) with which it forms a complex (Gaur *et al.*, 1991; Bai *et al.*, 1993; Lewis *et al.*, 1998). To investigate the effects of mutations in these genes on biofilm formation, we created and introduced null mutations of *sinI* and *sinR* into the wild strain 3610 and examined both pellicle formation in standing liquid medium and the formation of fruiting bodies on solid medium. We report that the *sinI* mutation blocked biofilm formation and that, oppositely to *sinI*, the *sinR* mutation caused the formation of robust, rugose, multicellular structures. Helping to explain the role of *sinI* and *sinR*, we show that SinR binds to the promoter region of the *eps* operon, thus repressing transcription of genes believed to be responsible for production of the EPS component of the extracellular matrix. Finally, we show that *sinI* as well as *yjbF* and *ymcA* are members of a pathway(s) that is responsible for counteracting SinR-mediated repression and thereby activating the *eps* operon and other genes involved in biofilm and fruiting body formation.

Results

SinI and *SinR* have opposing effects on the formation of multicellular communities

The results of Fig. 1 show that in liquid medium, the *sinR* mutant derivative of the wild strain 3610 grew as bundled chains and formed robust rugose pellicles. These structures were hardy and difficult to disrupt mechanically when probed with a toothpick. In contrast, the *sinI* mutant lacked bundled chains and was completely defective (at least initially; see below) in biofilm formation (Fig. 1). On solid medium, the *sinR* mutant formed extremely rough colonies in which it was difficult to discern individual fruiting bodies (Fig. 1). The *sinI* mutant, on the other hand, produced flat, featureless colonies devoid of fruiting bodies (Fig. 1). Consistent with the idea that SinI is an antagonist of SinR, the phenotype of a *sinI sinR* double mutant, both in liquid and on solid medium, was indistinguishable from that of a *sinR* mutant (Fig. 1).

Despite the severe effect of the *sinI* mutation in blocking biofilm formation, upon prolonged incubation in liquid

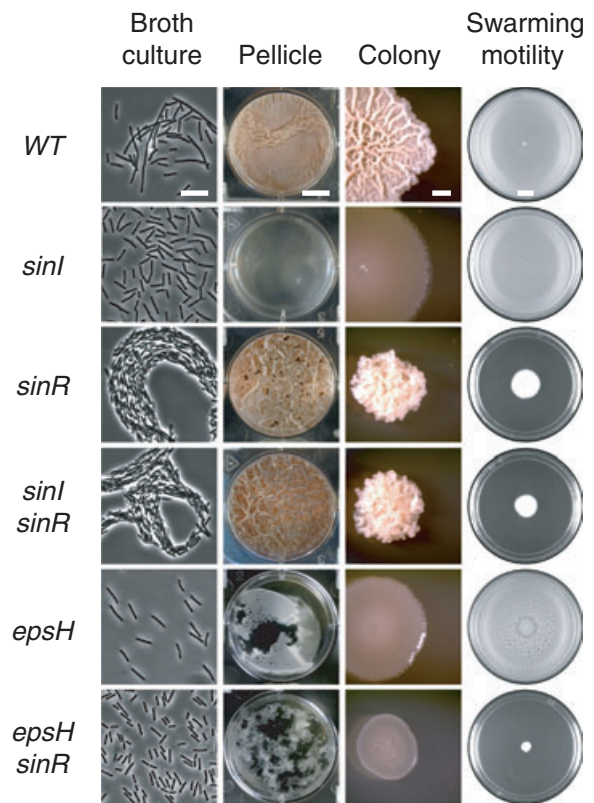


Fig. 1. Effect of mutations in *sinR*, *sinI* and *epsH* on chain bundling, pellicle formation, colony surface architecture and swarming motility. 'Broth culture' column depicts 1000 \times phase contrast images of cells grown to mid-exponential phase in LB broth and reveals effects of mutations in the indicated genes on the bundling of chains of cells. Scale bar is 10 μ m. 'Pellicle' column depicts top-down images of microtitre wells (6-well plate) in which cells have been grown in MSgg medium for 3 days at 22 $^{\circ}$ C and reveals effects of mutations on pellicle formation. Scale bar is 1 cm. 'Colony' column depicts 10 \times images of individual colonies grown on MSgg medium for 3 days at 22 $^{\circ}$ C and reveals effects on colony surface architecture. Scale bar is 1 mm. 'Swarming motility' column depicts top-down images of Petri plates containing LB and 0.7% agar, centrally inoculated and incubated at 37 $^{\circ}$ C overnight. Swarm plates were filmed against a black background such that zones of bacterial colonization appear white and uncolonized agar appears black. Scale bar is 1 cm. The indicated wild-type and mutant strains were as follows: WT (3610), *sinI* (DS91), *sinR* (DS92), *sinI sinR* (DS93), *epsH* (DS76) and *epsH sinR* (DS207).

medium the *sinI* mutant eventually produced a thick pellicle (Fig. 2A). The delay in pellicle formation led us to suppose that the *sinI* mutant eventually acquired suppressor mutations that restored its capacity to form a biofilm. In support of this hypothesis, cells that were isolated from the mutant biofilm, clonally purified and tested on solid medium, were found to exhibit an extremely rough phenotype that strongly resembled that of a *sinR* mutant, rather than the smooth, featureless phenotype of the original *sinI* mutant (Fig. 2B). Given that mutations in *sinR* suppress the effects of a *sinI* mutation (Fig. 1), one possibility was that the spontaneous suppressors were second-site mutations within *sinR*. To investigate this possibility, we

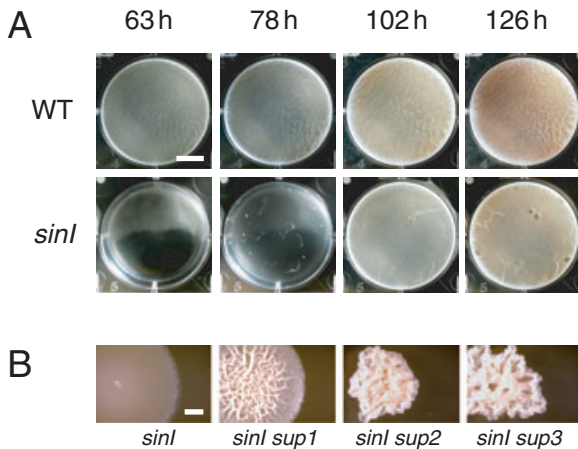


Fig. 2. Prolonged incubation leads to the appearance of suppressors that restore biofilm formation to a *sinI* mutant.

A. WT (3610) and *sinI* (DS91) mutant cells were grown in MSgg medium at 22°C for the indicated times. Notice that by 102 h the *sinI* mutant had formed a pellicle. Scale bar is 1 cm.

B. Surface features of colonies grown on MSgg medium. Shown are colonies of a *sinI* mutant (DS91) and three independently isolated suppressor strains derived from pellicles of the *sinI* mutant at 126 h: *sinI sup1* (FC139), *sinI sup2* (FC140) and *sinI sup3* (FC141). Scale bar is 1 mm.

amplified and sequenced the *sinR* gene from three independently arising *sinI* suppressor mutants. In each case, the mutants harboured (different) frameshift mutations within the *sinR* open reading frame (ORF) (Fig. 3). Interestingly, each of the spontaneous *sinR* frameshift mutations lie within, or adjacent to, a homopolymeric track of repetitive nucleotides. Tracks such as these are subject to high frequency mutation resulting from slipped-strand mispairing during DNA replication and have recently been determined to be responsible for phase regulation of the *B. subtilis* swarming motility gene, *swrA* (Levinson and Gutman, 1987; Kearns *et al.*, 2004). The nature of the spontaneous *sinR* mutations raises the possibility that, like swarming motility, biofilm formation in *B. subtilis* is also regulated by phase variation.

Surface motility and the formation of multicellular communities are alternative physiological states

In addition to the formation of multicellular communities, wild strains, but not standard laboratory strains, exhibit a second multicellular behaviour called swarming motility (Kearns and Losick, 2003). Swarming is a form of motility that takes place on solid medium in which small groups of cells (rafts) migrate along the surface of a substratum. As the swarm expands, a second internal population arises that is enriched in chains of non-motile cells. As mutations in *sinR* and *sinI* had profound effects on biofilm and fruiting body formation, we wondered whether the

mutations might also influence swarming motility. The *sinR* mutant was blocked in swarming motility (Fig. 1), a finding consistent with previous observations that *sinR* mutants are non-motile (Fein, 1979; Pooley and Karamata, 1984; Sekiguchi *et al.*, 1990). In contrast, the *sinI* mutant swarmed as readily as the wild type but lacked the characteristic population of chains of non-motile cells observed in the centre of swarms of the wild-type parent (Fig. 1; Fig. S1). Once again, the effects of the *sinR* mutation were epistatic to those of the *sinI* mutation (Fig. 1).

In summary, our results are consistent with the idea that *B. subtilis* exists in two mutually exclusive, physiological states in which cells either grow as bundled chains or as single motile cells. Each state is associated with an alternative multicellular behaviour in that growth in bundled chains promotes biofilm and fruiting body formation, whereas growth as single cells promotes swarming. We propose that SinR and SinI play a central role in regulating the switch between these two states and in this capacity determine which multicellular behaviour is adopted by the cells.

SinR is a negative regulator of genes involved in EPS synthesis

In earlier work we found that cells that contained mutations within the *eps* operon were defective in bundling of cell chains and produced fragile pellicles that would fre-

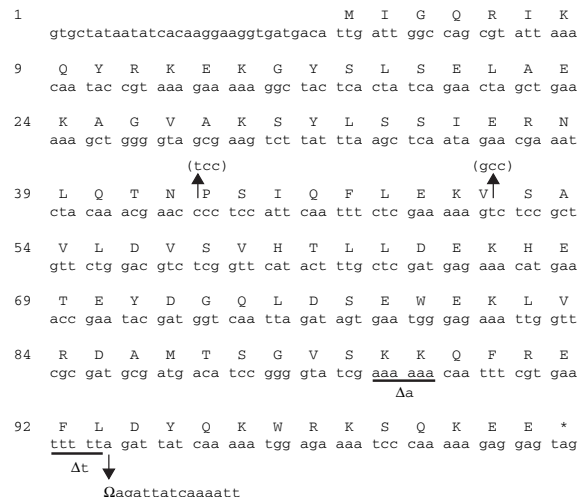


Fig. 3. Suppressor mutations in *sinR* that restore biofilm formation to *sinI*, *ybfF* and *ymcA* mutants. DNA sequence of the *sinR* ORF is given in lower case. The SinR amino acid sequence is in upper case above the DNA sequence. Mutations indicated below the DNA sequence are suppressors of a *sinI* mutation: deletion of an A:T base pair within the underlined stretch of A:T base pairs (FC139), deletion of a T:A base pair with the underlined stretch of T:A base pairs (FC140) and a duplication of the sequence indicated by the downward pointing arrow (FC141). Mutations indicated above the DNA sequence by the upward arrows are missense suppressors of *ybfF* (P42S, SSB581) and *ymcA* (V50A, SSB584) mutations.

Table 1. SinR represses expression of the *eps* operon.

Genotype ^a	Activity (MU) ^b
Wild type	30 ± 2
$\Delta epsH$	27 ± 3
$\Delta sinR \Delta epsH$	674 ± 92
$\Delta sinI \Delta epsH$	1 ± 0.4
$\Delta ylbF$	4 ± 0.7
$\Delta ymcA$	4 ± 0.3

a. The following P_{epsA} -*lacZ*-containing strains were used: FC5, FC13, FC14, FC15, DS698, DS699.

b. β -Galactosidase activity, presented as Miller units (MU), was measured for mid-exponential phase cells and is the average of three replicas.

quently shatter and sink to the bottom of the culture vessel. To investigate the relationship of *eps* genes to SinR, a mutation of *epsH* (formerly *yveR*) was introduced into a SinR mutant, which, as described above, is locked in the state of forming bundled chains. Consistent with the idea that the *eps* operon produces a substance involved in bundling, the *epsH* mutation was epistatic to the *sinR* mutation in that the double mutant resembled the *epsH* single mutant with respect to its inability to form bundled chains, pellicles, or architecturally complex colonies (Fig. 1). These data indicate that the *eps* genes (or at least *epsH*) act downstream of SinR and raise the possibility that SinR is a negative regulator of the expression of the *eps* operon. Interestingly, the *epsH* mutation did not restore swarming to the SinR mutant, a finding that indicates that the motility defect caused by the absence of SinR is not simply a consequence of tethering the cells to each other by extracellular polysaccharides (Fig. 1).

To investigate the idea that SinR is a negative regulator of the *eps* operon, we constructed a transcriptional fusion of *lacZ* to the promoter region (P_{epsA}) upstream of the first gene in the *eps* operon (*epsA*), and inserted the construct into the chromosome at the *amyE* locus (*amyE*:: P_{epsA} -*lacZ*). As the absence of SinR causes constitutive bundling, cells of a *sinR* mutant tend to clump, interfering with efforts to measure cell number and gene expression. To alleviate this problem, a mutation in the *epsH* gene was introduced into the reporter strain to ensure dispersed growth even in the presence of the *sinR* mutation. The *epsH* mutation had no effect on expression of the P_{epsA} -*lacZ* reporter and hence did not interfere with our efforts to investigate the influence of a *sinR* mutation on expression of the reporter (Table 1). We found that in medium (MSgg) that promoted biofilm formation, the *sinR* mutation increased expression of the P_{epsA} -*lacZ* construct by greater than 20-fold (Table 1). A *sinI* mutation, in contrast, had the opposite effect, reducing P_{epsA} -*lacZ* expression by at least 20-fold. We conclude that SinR is a potent negative regulator of the expression of the *eps* operon.

SinR binds to the regulatory region for the *eps* operon

To determine whether SinR represses the *eps* operon by directly binding to its regulatory region, we carried out electrophoretic mobility shift assays (EMSAs) with purified SinR and several promoter-containing DNA fragments (see *Experimental procedures*). The results presented in Fig. 4 show that SinR retarded the electrophoretic mobility of P_{epsA} -containing DNA, and did so with an apparent binding affinity similar to that for DNA containing the promoter region for *aprE*, a known direct target of SinR (Fig. 4A, Gaur *et al.*, 1991). Interestingly, SinR generated several species of P_{epsA} -containing DNA with reduced electrophoretic mobility, suggesting that the repressor binds to multiple sites within the *epsA* promoter region. As a negative control, little or no retardation in electrophoretic mobility was observed for DNA containing the

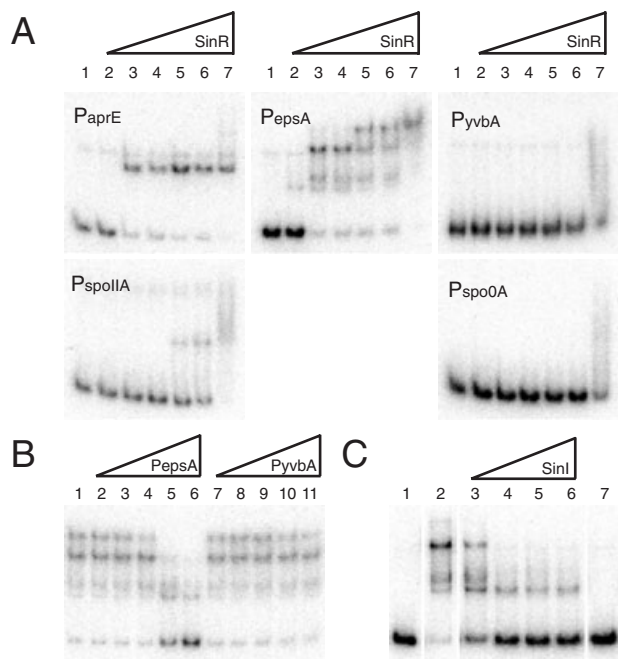


Fig. 4. SinR binds to DNA containing the promoter region for the *eps* operon.

A. Electrophoretic mobility shift assays (EMSAs) in which radiolabelled DNAs (indicated in the upper left corner of each panel) were mixed with purified SinR at the following concentrations: 0 nM (lane 1), 4 nM (lane 2), 10 nM (lane 3), 40 nM (lane 4), 100 nM (lane 5), 400 nM (lane 6) and 1 μM (lane 7).

B. EMSA experiment in which 40 nM of SinR was mixed with either unlabelled P_{epsA} -containing DNA (lanes 2–6) or unlabelled P_{yvbA} -containing DNA (lanes 7–11) added at 1× (lanes 2 and 7), 4× (lanes 3 and 8), 10× (lanes 4 and 9), 40× (lanes 5 and 10), and 100× (lanes 6 and 11) concentration relative to the concentration of radiolabelled P_{epsA} (100 nM).

C. EMSA experiment in which 40 nM of SinR was mixed with its antagonist SinI at the following concentrations: 0 nM (lane 2), 10 nM (lane 3), 40 nM (lane 4), 100 nM (lane 5) and 400 nM (lane 6) prior to the addition of radiolabelled P_{epsA} -containing DNA (100 nM). Lane 1 had P_{epsA} -containing DNA without added proteins and lane 7 had a mixture of P_{epsA} -containing DNA and 400 nM SinI but no SinR. All lanes for panel C were taken from the same gel.

promoter for an arbitrarily chosen gene (*yvbA*) not known nor believed to be under SinR control (Fig. 4A). As a further indication of a specific interaction with the radioactive P_{epsA} -containing DNA, SinR binding was inhibited by the addition of an excess of unlabelled P_{epsA} -containing competitor DNA, but not by an excess of P_{yvbA} -containing DNA (Fig. 4B). SinR has also been reported to bind to the promoter regions for the sporulation genes *spo0A* and *spoIIA* (Mandic-Mulec *et al.*, 1992; 1995). However, we detected little or no effect on the mobility of P_{spo0A} -containing DNA and only a weak effect on P_{spoIIA} -containing DNA (Fig. 4A). If these sporulation genes are indeed direct targets of SinR, then they evidently have much weaker binding sites for the repressor than do *epsA* and *aprE*.

SinI is an antagonist of SinR that inhibits the capacity of the repressor to bind to its targets, such as the promoter region of *aprE* (Bai *et al.*, 1993). To determine if SinI would similarly interfere with the binding of SinR to P_{epsA} -containing DNA, SinR was mixed with various concentrations of purified SinI and then tested for DNA-binding activity by EMSA. As the amount of SinI added to the reaction was increased, the ability of SinR to retard the electrophoretic mobility of DNA containing P_{epsA} was diminished (Fig. 4C). This was particularly evident from the accumulation of the unbound (unretarded) labelled probe, which was almost entirely depleted in the absence of the antagonist. Nevertheless, even at a 10-fold excess of SinI to SinR, some binding to P_{epsA} -containing DNA could still be detected by the persistence of a species with retarded mobility (Fig. 4C, lane 6). SinI alone had no effect on the electrophoretic mobility of P_{epsA} -containing DNA (Fig. 4C, lane 7).

Before attempting to localize the precise binding site(s) for SinR within the *eps* regulatory region, we carried out primer extension analysis to map the transcriptional start site for the operon. The results show that the start site, which is designated as position +1, was located 56 bp upstream of the first gene in the operon (Fig. 5B). The canonical recognition sequences for promoters used by RNA polymerase containing the housekeeping sigma factor σ^A are TATAAT for the '-10 sequence' and TTGACA for the '-35 sequence' (Moran *et al.*, 1982). *B. subtilis* promoters frequently have an AT-rich sequence or UP element located just upstream of the -35 sequence (Ross *et al.*, 1993; Fredrich *et al.*, 1995). Centred at position -10 relative to the transcriptional start site is a perfect match (TATAAT) to the canonical -10 sequence and centred at position -32 is a sequence (TTTTAA) that somewhat (three out of six positions) conforms to a canonical -35 sequence. We also note the presence of an AT-rich sequence just upstream of the putative -35 sequence that could correspond to a UP element.

To localize the binding site(s) for SinR in the *eps* regulatory region, we carried out footprinting experiments in which radiolabelled P_{epsA} -containing DNA was mixed with

various concentrations of SinR. Next, the DNA–SinR complexes were treated with DNase I, and the digestion products were subjected to electrophoresis. The results show that SinR protected two regions of P_{epsA} from the action of DNase I (See Fig. 5A). The region proximal to *epsA* contained an inverted repeat of the sequence GTTCTCT centred at positions -58 and -67 relative to the transcriptional start site. The extended region of protection distal to *epsA* contained three nearly identical direct repeats of the same sequence centred at positions -129, -150 and -159 relative to the transcriptional start site. We propose that these are operator sites for SinR, and that the motif GTTCTCT is the recognition sequence for SinR, which differs from that previously published (Shafikhani *et al.*, 2002). We note the presence of a similar sequence (GTTCTCA) in the binding site for SinR within the regulatory region for *aprE* (Gaur *et al.*, 1991). Both regions of SinR binding are too far upstream to compete with the binding of RNA polymerase to the -10 and -35 elements of the *eps* promoter. Conceivably, SinR acts by occluding the UP element or by preventing the binding of an unknown activator protein.

Mutations in sinR bypass genes required for biofilm formation

We recently identified two genes of unknown function, *yIbF* and *ymcA*, that are required for biofilm formation (Tortosa *et al.*, 2000; Branda *et al.*, 2004). Consistent with the flat featureless colonies produced by *yIbF* and *ymcA* mutants, mutation of either gene resulted in a marked reduction in *eps* gene expression in both liquid and solid medium (Table 1, Fig. 6A). As indicated above, a mutation in *sinR* reverses the block in biofilm formation caused by a mutation in *sinI*. We therefore wondered whether a *sinR* mutation would similarly bypass the requirement for *yIbF* or *ymcA* in biofilm formation. Whereas single mutants of each of the aforementioned genes produced flat featureless colonies, the introduction of a *sinR* mutation restored the formation of colonies with complex surface features (Fig. 6) and rescued the ability of the mutants to form robust pellicles in standing liquid minimal medium (data not shown).

Reinforcing the view that *yIbF* and *ymcA* lie upstream of *sinR* are the following observations concerning the appearance of spontaneous suppressors of mutations in *yIbF* and *ymcA*. Whereas *yIbF* and *ymcA* mutants in standing liquid medium were initially defective in pellicle formation, prolonged incubation resulted in the appearance of suppressor mutants that had regained the ability to form a pellicle. Moreover, after clonal purification, these suppressor mutants were found to produce colonies with complex architectural features (Fig. 6B). Suspecting that the suppressors had acquired second-site mutations in

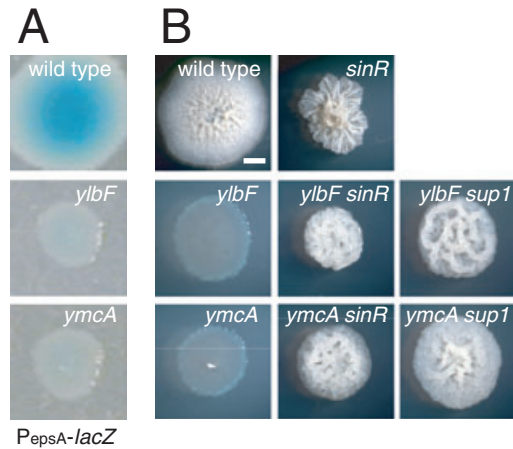


Fig. 6. A *sinR* mutation restores complex colony architecture to mutants blocked in biofilm formation.
 A. Colonies of *P_{epsA-lacZ}*-containing strains FC5 (wild type), DS698 (*ylbF*) and DS699 (*ymcA*) grown on MSgg medium supplemented with Xgal to detect β -galactosidase activity.
 B. Colonies of strains 3610 (wild type), DS92 (*sinR*), SSB136 (*ylbF*), SSB561 (*ylbF sinR*), SSB581 (*ylbF sup1*), SSB132 (*ymcA*), SSB563 (*ymcA sinR*) and SSB584 (*ymcA sup1*). SSB581 and SSB563 are suppressor mutant isolates obtained from pellicles arising after prolonged incubation of the *ylbF* and *ymcA* mutants. Scale bar is 1 mm.

sinR (as we had observed for similar suppressors of *sinI*), we amplified by polymerase chain reaction (PCR) and sequenced *sinR* from each of the suppressor mutants. In both cases, the suppressors were found to contain a missense mutation in the ORF for *sinR* (Fig. 3). The simplest conclusion from these findings is that like *sinI*, the genes *ylbF* and *ymcA* lie upstream of *sinR* in a pathway that is responsible for reversing SinR-mediated repression of the *eps* operon.

Discussion

The results of this investigation lead us to conclude that SinR is a central regulator in the assembly of *B. subtilis* cells into multicellular communities. SinR plays this role by repressing the transcription of the *eps* operon, which directs EPS biosynthesis, as well as other, yet-to-be-identified target genes. EPS is, in turn, responsible for the adhesion of chains of cells into bundles. These bundles appear essential to give rise to complex architectural features of biofilms, such as fruiting bodies.

We further propose that activation of the *eps* operon (and other SinR targets) requires the action of SinI, YlbF and YmcA, which are collectively responsible for reversing, or otherwise counteracting, SinR-mediated repression. SinI antagonizes the binding of SinR to DNA *in vitro* (Bai *et al.*, 1993; Lewis *et al.*, 1998), as confirmed by the results presented herein. However, our genetic analyses indicate that, while necessary, SinI was insufficient to fully overcome SinR-mediated repression *in vivo*. High-level

expression of the *eps* operon additionally required the action of YlbF and YmcA. These proteins are similar to each other in their amino acid sequences, and it is appealing to imagine that they interact to form a heteromeric complex. Conceivably, YlbF and YmcA help to counteract SinR-mediated repression and do so by interacting (separately or as a heteromeric complex) with SinI or SinR (or both) or by stimulating the synthesis or stability of SinI. Alternatively, YlbF and YmcA could influence the expression of the *eps* operon in some other indirect manner. Indeed, YlbF and YmcA mutants display some pleiotropy in that they grow slightly more slowly than the wild type (S. S. Branda, D. B. Kearns, R. Losick and R. Kolter, unpubl. results).

Interestingly, *sinI* is under the positive control of the sporulation regulatory proteins Spo0A and σ^H (Gaur *et al.*, 1988; Shafikhani *et al.*, 2002). Previous work has shown that mutations in the genes (*spo0A* and *spo0H*, respectively) for Spo0A and σ^H block biofilm and fruiting body formation (Branda *et al.*, 2001; Hamon and Lazazzera, 2001). In light of the role assigned to SinI in the present investigation, we suppose that Spo0A and σ^H contribute to multicellularity in part by stimulating the synthesis of the SinR antagonist. Hamon and Lazazzera (2001) have reported that the requirement for Spo0A in biofilm formation can be bypassed by a mutation in *abrB*, a repressor gene that is under the negative control of Spo0A. We have found that a mutation in *sinI* is epistatic to an *abrB* mutation (that is, an *abrB* mutation did not relieve the requirement for *sinI* in biofilm formation in an *abrB sinI* double mutant; D. B. Kearns, S. S. Branda, R. Kolter and R. Losick, unpubl. results). We therefore conclude that, like YlbF, YmcA and SinI, AbrB somehow acts to counteract SinR-mediated repression.

Finally, we note that SinR has traditionally been studied in the context of domesticated, laboratory strains, which are incapable of swarming motility and form neither robust pellicles in standing liquid medium nor architecturally complex colonies on solid medium. Such studies have implicated SinR in diverse processes, such as genetic competence (Hahn *et al.*, 1994; Liu, L. *et al.*, 1996), production of autolysins (Sekiguchi *et al.*, 1988; Kuroda and Sekiguchi, 1993; Rashid and Sekiguchi, 1996), production of secreted proteases (Gaur *et al.*, 1991; Bai *et al.*, 1993; Olmos *et al.*, 1997), swimming motility (Fein, 1979; Pooley and Karamata, 1984; Sekiguchi *et al.*, 1990; Barilla *et al.*, 1994; Márquez-Magaña *et al.*, 1994; Fredrick and Hellmann, 1996) and sporulation (Gaur *et al.*, 1986; Mandic-Mulec *et al.*, 1992; Louie *et al.*, 1992; Cervin *et al.*, 1998). The direct targets of SinR action in most of these cases are unknown, and the overall physiological significance of SinR has been difficult to elucidate. In the context of wild *B. subtilis* strains, however, SinR can be seen as a master regulator that governs entry into two alternative, and

mutually exclusive, physiological states: a motile state in which the bacteria are capable of swimming as single cells in liquid or swarming in groups of cells on surfaces, and a non-motile state in which the bacteria form sessile, multicellular communities.

Experimental procedures

Strains and growth conditions

Bacillus subtilis PY79, 168 and 3610 were grown in Luria–Bertani (LB) 10 g tryptone, 5 g yeast extract, 5 g NaCl per L broth or LB plates supplemented with 1.5% agar at 37°C. For pellicle formation experiments, 12 µl mid-log phase culture was inoculated into 12 ml minimal MSgg medium (5 mM potassium phosphate, pH 7, 100 mM MOPS, pH 7, 2 mM MgCl₂, 700 µM CaCl₂, 50 µM MnCl₂, 50 µM FeCl₃, 1 µM ZnCl₂, 2 µM thiamine, 0.5% glycerol, 0.5% glutamate, 50 µg ml⁻¹ tryptophan, 50 µg ml⁻¹ phenylalanine and 50 µg ml⁻¹ threonine) and incubated at 22°C (Branda *et al.*, 2001). For colony architecture analysis, colonies were toothpick inoculated onto minimal MSgg medium fortified with 1.5% Bacto agar and incubated at 22°C. When appropriate, antibiotics were included at the following concentrations: 10 µg ml⁻¹ tetracycline, 100 µg ml⁻¹ spectinomycin, 5 µg ml⁻¹ chloramphenicol, 5 µg ml⁻¹ kanamycin, and 1 µg ml⁻¹ erythromycin plus 25 µg ml⁻¹ lincomycin (ml).

'Swarm agar' plates (25 ml) containing LB fortified with 0.7% agar were prepared fresh and the following day were dried for 30 min in a laminar flow hood. Each plate was toothpick inoculated from an overnight colony and scored for swarming motility after 24 h incubation at 37°C (Kearns and Losick, 2003). Plates were visualized with a Bio-Rad geldoc system and digitally captured using Bio-Rad Quantity One software. A tripod mounted Sony CoolPix950 digital camera was used to capture swarm images illuminated by an oblique transmitted light source.

The handling of *sinI*, *yhbF* and *ymcA* mutants required special care as prolonged incubation in standing liquid minimal medium or as colonies on solid minimal medium readily led to outgrowth of suppressor mutants. As a precaution, we frequently returned to frozen stocks of our strains and conducted genetic backcrosses when necessary to ensure that strains were free of suppressor mutations.

Strain construction

All insertion deletion mutations were generated using long flanking homology PCR (using primers indicated in Table S2) and transformed into competent cells of strain PY79 (Wach, 1996). DNA containing a spectinomycin drug resistance gene (pDG1726) was used as a template for marker replacement (Guérout-Fleury *et al.*, 1995). Mutations were transferred to the 3610 background using SPP1 mediated generalized transduction (Yasbin and Young, 1974). All strains used in this study are listed in Table S1.

SPP1 phage transduction

To 0.2 ml of dense culture grown in TY broth (LB broth sup-

plemented after autoclaving with 10 mM MgSO₄ and 100 µM MnSO₄), serial dilutions of SPP1 phage stock were added and statically incubated for 15 min at 37°C. To each mixture, 3 ml of TYSA (molten TY supplemented with 0.5% agar) was added, poured atop fresh TY plates, and incubated at 37°C overnight. Top agar from the plate containing near confluent plaques was harvested by scraping into a 50 ml conical tube, vortexed, and centrifuged at 5000 g for 10 min. The supernatant was treated with 25 µg ml⁻¹ DNase final concentration before being passed through a 0.45 µm syringe filter and stored at 4°C.

Recipient cells were grown to stationary phase in 2 ml of TY broth at 37°C. A total of 0.9 ml of cells were mixed with 10 µl of SPP1 donor phage stock. A total of 9 ml of TY broth was added to the mixture and allowed to stand at 37°C for 30 min. The transduction mixture was then centrifuged at 5000 g for 10 min, the supernatant was discarded and the pellet was resuspended in the remaining volume. A total of 100 µl of cell suspension was then plated on TY fortified with 1.5% agar, the appropriate antibiotic, and 10 mM sodium citrate.

Sequencing *sinR*

A PCR product containing the *sinR* gene was amplified from *B. subtilis* chromosomal DNA (either from strain 3610 or the appropriate suppressor strain) using the primers -33 *sinR* F/+512 *sinR* R. The *sinR* PCR product was then sequenced using either primer individually.

Reporter and protein expression constructs

All primers used in the construction of plasmids are listed in Table S2. To generate the P_{epsA}-*lacZ* reporter construct pFC1, a PCR product containing the P_{epsA} promoter was amplified from *B. subtilis* 3610 chromosomal DNA using primers PepsAF and PepsAR. The PCR product was cloned into the *EcoRI* and *BamHI* sites of plasmid pDG268, which carries a chloramphenicol-resistance marker and a polylinker upstream of the *lacZ* gene between two arms of the *amyE* gene (Antoniewski *et al.*, 1990).

To generate plasmids for the expression of N-terminal 6-histidine translation fusions to SinR and SinI (pDP90 and pDP91), PCR products containing the *sinR* and *sinI* genes were amplified from *B. subtilis* 3610 chromosomal DNA using primers H6sinRF/H6sinRR and H6sinIF/H6sinIR respectively. The PCR products were then cloned into the *NheI/XhoI* sites of pET28a(+) (Novagen).

β-Galactosidase assay

One millilitre of cells were harvested from a mid-log phase (OD₆₀₀ ~0.5) culture grown in MSgg broth shaken at 37°C, harvested and resuspended in an equal volume of Z buffer (40 mM NaH₂PO₄, 60 mM Na₂HPO₄, 1 mM MgSO₄, 10 mM KCl and 38 mM β-mercaptoethanol). To each sample, lysozyme was added to a final concentration of 0.2 mg ml⁻¹ and incubated at 30°C for 15 min. Each sample was diluted appropriately in 500 µl of Z buffer and the reaction was started with 100 µl of 4 mg ml⁻¹ 2-nitrophenyl β-D-galactopy-

ranoside (in Z buffer) and stopped with 250 μ l 1 M Na_2CO_3 . The OD_{420} of the reaction mixtures was recorded and the β -galactosidase specific activity was calculated according to the equation: $[\text{OD}_{420}/\text{time} \times \text{OD}_{600}] \times \text{dilution factor} \times 1000$.

Protein purification

Plasmids pDP90 and pDP91 were transformed into the Invitrogen overexpression strain 'CodonPlus', a BL21 DE3 derivative carrying a plasmid bearing three rare codons (arginine, isoleucine and leucine) for optimal expression of heterologous proteins. A total of 500 ml of culture was grown in LB broth supplemented with 25 $\mu\text{g ml}^{-1}$ kanamycin and 50 $\mu\text{g ml}^{-1}$ chloramphenicol at 30°C until an OD_{600} of 0.5 was obtained, at which point IPTG was added to a final concentration of 1 mM. The cultures were incubated for 2 h at 30°C, washed once in 25 ml of PBS buffer, harvested and resuspended in 18 ml of lysis/binding buffer (50 mM Tris HCl, 500 mM NaCl and 10 mM imidazole, pH 8.5). Two millilitres of Novagen bug buster lysis solution and 10 μ l of Novagen benzonase nuclease (25 U μl^{-1}) was added to the suspension, and rotated end over end for 1 h at room temperature. The lysate was centrifuged at 5000 r.p.m. for 5 min to remove most of the cell debris and then the supernatant was ultracentrifuged at 35 000 r.p.m. for 30 min at 4°C.

One millilitre of Ni-NTA agarose beads (Qiagen) was added to the cleared lysate and rotated for 1 h at 4°C. The lysate/bead mixture was then loaded onto a column and washed five times, each with two bed volumes of wash buffer (50 mM Tris HCl, 500 mM NaCl and 20 mM imidazole, pH 8.5). The beads were recollected in 1 ml of elution buffer (10 mM Tris HCl, 10 mM MgCl_2 , 1 mM EDTA, 0.3 mM DTT, 5% glycerol, 1 mM PMSF, pH 8.5) (Gaur *et al.*, 1991), to which 3 μ l of 1.4 U μl^{-1} biotinylated thrombin (Novagen) was added and rotated at room temperature overnight. The eluate was recovered by passing the bead slurry over a fresh column.

To remove the biotinylated thrombin, 130 μ l of streptavidin agarose was added to the protein-containing supernatant, and rotated for 1 h at room temperature. The supernatant was recovered by passing over a fresh column. Finally, the protein samples were dialysed against dialysis buffer (10 mM Tris HCl, 10 mM MgCl_2 , 1 mM EDTA, 0.3 mM DTT, 50% glycerol, 1 mM PMSF, pH 8.5), aliquoted and stored at -80°C.

Electrophoretic mobility shift assay (EMSA)

DNA probes were generated by PCR using chromosomal DNA from *B. subtilis* 3610 and the following primer combinations: PepsAF2/PepsAR2 (P_{epsA} probe), PaprEF/PaprER (P_{aprE} probe), ECH245/ECH246 (P_{yvbA} probe), omf187/omf188 (P_{spolIA} probe) and Pspo0AF/Pspo0AR (P_{spo0A} probe). Each probe was gel purified and 5' end labelled with 10 μCi of [γ - ^{32}P]-ATP (NEG002A, New England Nuclear) and polynucleotide kinase (New England Biolabs). DNA binding reactions were conducted in 30 μ l of binding buffer (10 mM Tris HCl, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, 10 $\mu\text{g ml}^{-1}$ BSA) containing 25 $\mu\text{g ml}^{-1}$ polydeoxyinosinic-deoxycytidylic acid (poly dI-dC). Various concentrations of SinR were added to approximately 100 nM radiolabelled DNA probe and incubated for 20 min at room temperature. A

total of 10 μ l of each binding reaction was loaded on a 6% polyacrylamide 0.5 \times TBE gel and resolved for 1 h at 200 mV. When appropriate, SinI and SinR were combined and incubated for 15 min at room temperature prior to the addition of radiolabelled probe.

Primer extension assay

Total RNA was isolated from mid-log *B. subtilis* 3610 cells grown in MSgg liquid medium. RNA was isolated using the hot acid/phenol method (protocol available at <http://mcb.harvard.edu/losick/fawcettpaper/RNAprep.htm>).

The primer extension product was obtained using the primer + 94epsAR (Table S2) 5' end labelled with 40 μCi [γ - ^{32}P]-ATP and polynucleotide kinase. The radiolabelled primer (0.2 pmol) was annealed to 20 μg of total RNA in a 10 μ l reaction volume of 1 \times first strand buffer (Invitrogen SuperScript First Strand Synthesis System for RT PCR). The annealing reaction was heated to 95°C for 1 min, transferred to 70°C for 10 min, and then put on ice for 2 min. The extension reaction was carried out using 5 μ l of the annealing reaction, 0.01 M DTT, 1 mM dNTP's, 1 \times first strand buffer and 10 units of Invitrogen SuperScript II RNase H⁻ Reverse Transcriptase. The reaction was incubated at 45°C and stopped with 5 μ l of formamide loading buffer (80% deionized formamide, 10 mM EDTA, 1 mg ml^{-1} xylene cyanol FF, 1 mg ml^{-1} bromophenol blue).

A sequencing ladder was generated to a DNA fragment generated by PCR using primers -565 epsAF/+94epsAR (Table S2). A total of 2 μ l of PCR product was denatured in 0.2 N NaOH and 100 μM EDTA for 5 min at room temperature before adding ammonium acetate to a final concentration of 0.8 M. The DNA was ethanol precipitated and resuspended in 7 μ l of water, to which sequenase (USB) reaction buffer and 0.4 pmol of radiolabelled primer were added. The mixture was heated to 65°C for 2 min and cooled to room temperature for 10 min. To the annealing mix, 2 μ l 1.5 μM dNTP's, 1 μ l 0.1 M DTT, and 3.2 units of sequenase (USB) were added. The reactions were incubated at room temperature for 90 s. A total of 3.5 μ l of the reaction was transferred to tubes containing 200 pmol dNTP's with 20 pmol of the appropriate ddNTP. After incubating the termination reactions at 37°C for 5 min, the reactions were stopped with 4 μ l of formamide loading buffer.

Prior to loading the reactions on an 8% sequencing gel, the primer extension and sequencing reactions were heated to 95°C for 5 min. A total of 7 μ l of the primer extension and 4 μ l of the sequencing reaction were resolved on the gel and visualized by phosphoimaging.

DNase I footprinting assay

Primer PepsAF2 (100 pmol) was 5' end labelled with 70 μCi of [γ - ^{32}P]-ATP and polynucleotide kinase. A PCR product containing the P_{epsA} promoter region was generated using 20 pmol radiolabelled PepsAF2 primer and 20 pmol unlabelled PepsAR2 primer using *B. subtilis* 3610 chromosomal DNA as a template. The PCR product was gel purified and subjected to scintillation counting such that, for each reaction, 30 000 c.p.m. of radiolabelled PCR product was mixed with

SinR protein in 100 μ l of footprinting buffer (20 mM Tris pH 8.0, 5 mM MgCl₂, 5 mM CaCl₂, 0.1 mM DTT, 0.1 mM EDTA, 50 μ g ml⁻¹ bovine serum albumin) containing 5 μ g ml⁻¹ poly dI-dC and incubated for 15 min at room temperature. To each mixture, 2 μ l of DNase I (1:50 dilution of 1 U μ l⁻¹ stock, Invitrogen) was added and incubated for 30 s at room temperature before digestion was inhibited by the addition of 25 μ l of stop solution (1.5 M sodium acetate pH 5.3, 20 mM EDTA and 400 μ g ml⁻¹ glycogen). Each reaction was ethanol precipitated and resuspended to a final volume of 8 μ l in formamide running buffer (80% deionized formamide, 10 mM EDTA, 1 mg ml⁻¹ xylene cyanol and 1 mg ml⁻¹ bromophenol blue). A total of 4 μ l of each sample was loaded on an 8% sequencing gel (SequaGel Sequencing System, National Diagnostics) and resolved for 2 h at 35 mW.

Acknowledgements

We thank D. Dubnau, members of the Losick and Kolter laboratories, and, in particular, M. Fujita, E. Hobbs, A. Handler and B. Gorbatyuk for helpful advice and discussions. This work was supported by an NIH National Research Service Award GM66612 to D.K., National Institutes of Health grants GM18568 to R.L. and GM58213 to R.K. and American Cancer Society postdoctoral fellowship PF0033201MBC and The Medical Foundation/Charles A. King Trust (Fleet National Bank) to S.S.B.

Supplementary material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4440/mmi4440sm.htm>

Fig. S1. Effect of a *sinI* mutation on the formation of a centrally located population of non-motile cells during swarming.

Table S1 Strains and plasmids.

Table S2 Primers.

References

Antoniewski, C., Savelli, B., and Stragier, P. (1990) The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. *J Bacteriol* **172**: 86–93.

Bai, U., Mandic-Mulec, I., and Smith, I. (1993) SinI modulates the activity of SinR, a developmental switch protein of *Bacillus subtilis*, by protein–protein interaction. *Genes Dev* **7**: 139–148.

Barilla, D., Caramori, T., and Galizzi, A. (1994) Coupling of flagellin gene transcription to flagellar assembly in *Bacillus subtilis*. *J Bacteriol* **176**: 4558–4564.

Branda, S.S., González-Pastor, J.E., Ben-Yehuda, S., Losick, R., and Kolter, R. (2001) Fruiting body formation by *Bacillus subtilis*. *Proc Natl Acad Sci USA* **98**: 11621–11626.

Branda, S.S., González-Pastor, J.E., Dervyn, E., Ehrlich, D., Losick, R., and Kolter, R. (2004) Genes involved in the formation of structured multicellular communities by *Bacillus subtilis*. *J Bacteriol* **186**: 3970–3979.

Cervin, M.A., Lewis, R.J., Brannigan, J.A., and Spiegel-

man, G.B. (1998) The *Bacillus subtilis* regulator SinR inhibits *spoIIIG* promoter transcription in vitro without displacing RNA polymerase. *Nucleic Acids Res* **26**: 3806–3812.

Cohn, F.E. (1872) Untersuchungen über Bakterien. *Beitr Biol Pflanz* **1**: 124–224.

Fein, J.E. (1979) Possible involvement of bacterial autolytic enzymes in flagellar morphogenesis. *J Bacteriol* **137**: 933–946.

Fredrick, K., and Helmann, J.D. (1996) FlgM is the primary regulator of σ^D activity, and its absence restores motility to a *sinR* mutant. *J Bacteriol* **178**: 7010–7013.

Fredrick, K., Caramori, T., Chen, Y.-F., Galizzi, A., and Helmann, J.D. (1995) Promoter architecture in the flagellar regulon of *Bacillus subtilis*: high-level expression of flagellin by the σ^D RNA polymerase requires an upstream promoter element. *Proc Natl Acad Sci USA* **92**: 2582–2586.

Gaur, N.K., Dubnau, E., and Smith, I. (1986) Characterization of a cloned *Bacillus subtilis* gene that inhibits sporulation in multiple copies. *J Bacteriol* **168**: 860–869.

Gaur, N.K., Cabane, K., and Smith, I. (1988) Structure and expression of the *Bacillus subtilis* *sin* operon. *J Bacteriol* **170**: 1046–1053.

Gaur, N.K., Oppenheim, J., and Smith, I. (1991) The *Bacillus subtilis* *sin* gene, a regulator of alternate developmental processes, codes for a DNA-binding protein. *J Bacteriol* **173**: 678–686.

Guérout-Fleury, A., Shazand, K., Frandsen, N., and Stragier, P. (1995) Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene* **167**: 335–336.

Hahn, J., Kong, L., and Dubnau, D. (1994) The regulation of competence transcription factor synthesis constitutes a critical control point in the regulation of competence in *Bacillus subtilis*. *J Bacteriol* **176**: 5753–5761.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2**: 95–108.

Hamon, M.A., and Lazazzera, B.A. (2001) The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. *Mol Microbiol* **45**: 1199–1209.

Hamon, M.A., Stanley, N.R., Britton, R.A., Grossman, A.D., and Lazazzera, B.A. (2004) Identification of AbrB-regulated genes involved in biofilm formation by *Bacillus subtilis*. *Mol Microbiol* **52**: 847–860.

Kearns, D.B., and Losick, R. (2003) Swarming motility in undomesticated *Bacillus subtilis*. *Mol Microbiol* **49**: 581–590.

Kearns, D.B., Chu, F., Rudner, R., and Losick, R. (2004) Genes governing swarming in *Bacillus subtilis* and evidence for a phase variation mechanism controlling surface motility. *Mol Microbiol* **52**: 357–369.

Kunst, F., Ogaswara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., et al. (1997) The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* **390**: 249–256.

Kuroda, A., and Sekiguchi, J. (1993) High-level transcription of the major *Bacillus subtilis* autolysin operon depends on expression of the Sigma D gene and is affected by a *sin* (*flaD*) mutation. *J Bacteriol* **175**: 795–801.

Levinson, G., and Gutman, G.A. (1987) Slipped-strand mis-

- pairing: a major mechanism for DNA sequence evolution. *Mol Biol Evol* **4**: 203–221.
- Lewis, R.J., Brannigan, J.A., Offen, W.A., Smith, I., and Wilkinson, A.J. (1998) An evolutionary link between sporulation and prophage induction in the structure of a repressor: anti-repressor complex. *J Mol Biol* **283**: 907–912.
- Liu, D., Cole, R.A., and Reeves, P.R. (1996) An O-antigen processing function for Wzx (RfbX): a promising candidate for O-unit flippase. *J Bacteriol* **178**: 2102–2107.
- Liu, L., Nakano, M.M., Lee, O.H., and Zuber, P. (1996) Plasmid-amplified *comS* enhances genetic competence and suppresses *sinR* in *Bacillus subtilis*. *J Bacteriol* **178**: 5144–5152.
- Louie, P., Lee, A., Stansmore, K., Grant, R., Ginther, C., and Leighton, T. (1992) Roles of *rpoD*, *spoIIF*, *spoIIJ*, *spoIIN*, and *sin* in regulation of *Bacillus subtilis* stage II sporulation-specific transcription. *J Bacteriol* **174**: 3570–3576.
- Mandic-Mulec, I., Gaur, N., Bai, U., and Smith, I. (1992) *Sin*, a stage-specific repressor of cellular differentiation. *J Bacteriol* **174**: 3561–3569.
- Mandic-Mulec, I., Doukhan, L., and Smith, I. (1995) The *Bacillus subtilis* *SinR* protein is a repressor of the key sporulation gene *spo0A*. *J Bacteriol* **177**: 4619–4627.
- Márquez-Magaña, L.M., Mirel, D.B., and Chamberlin, M.J. (1994) Regulation of σ^D expression and activity by *spo0*, *abrB*, and *sin* gene products in *Bacillus subtilis*. *J Bacteriol* **176**: 2435–2438.
- Moran, C.P., Jr, Lang, N., LeGrice, S.F., Lee, G., Stephens, M., Sonenshein, A.L., *et al.* (1982) Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. *Mol Gen Genet* **186**: 339–346.
- Olmos, J., de Anda, R., Ferrari, E., Bolivar, F., and Valle, F. (1997) Effects of the *sinR* and *degU32* (Hy) mutations on the regulation of the *aprE* gene in *Bacillus subtilis*. *Mol Gen Genet* **253**: 562–567.
- Pooley, K., and Karamata, D. (1984) Genetic analysis of autolysin deficient and flagellaless mutants of *Bacillus subtilis*. *J Bacteriol* **160**: 1123–1129.
- Rashid, M.H., and Sekiguchi, J. (1996) *flaD* (*sinR*) mutations affect SigD-dependent functions at multiple points in *Bacillus subtilis*. *J Bacteriol* **178**: 6640–6643.
- Rick, P.D., Barr, K., Sankaran, K., Kajimura, J., Rush, J.S., and Waechter, C.J. (2003) Evidence that the *wzxE* gene of *Escherichia coli* K-12 encodes a protein involved in the transbilayer movement of a trisaccharide-lipid intermediate in the assembly of enterobacterial common antigen. *J Biol Chem* **278**: 16534–16542.
- Ross, W., Gosink, K.K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., *et al.* (1993) A third recognition element in bacterial promoters: DNA binding by the α subunit of RNA polymerase. *Science* **262**: 1407–1413.
- Sekiguchi, J., Ezaki, B., Kodama, K., and Akamatsu, T. (1988) Molecular cloning of a gene affecting the autolysin level and flagellation in *Bacillus subtilis*. *J Gen Microbiol* **134**: 1611–1621.
- Sekiguchi, J., Ohsu, H., Kuroda, A., Moriyama, H., and Akamatsu, T. (1990) Nucleotide sequences of the *Bacillus subtilis* *flaD* locus and a *B. licheniformis* homologue affecting autolysin level and flagellation. *J Gen Microbiol* **136**: 1223–1230.
- Shafikhani, S.H., Mandic-Mulec, I., Strauch, M.A., Smith, I., and Leighton, T. (2002) Postexponential regulation of *sin* operon expression in *Bacillus subtilis*. *J Bacteriol* **184**: 564–571.
- Tatusov, R.L., Koonin, E.V., and Lipman, D.J. (1997) A genomic perspective on protein families. *Science* **278**: 631–637.
- Tortosa, P., Albano, M., and Dubnau, D. (2000) Characterization of *ylbF*, a new gene involved in competence development and sporulation in *Bacillus subtilis*. *Mol Microbiol* **35**: 1110–1119.
- Wach, A. (1996) PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* **12**: 259–265.
- Yasbin, R.E., and Young, F.E. (1974) Transduction in *Bacillus subtilis* by bacteriophage SPP1. *J Virol* **14**: 1343–1348.