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A Novel Regulatory Protein Governing Biofilm Formation in *Bacillus subtilis*

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Abstract

Production of an extracellular matrix is a hallmark of biofilm formation. In the spore-forming bacterium *Bacillus subtilis*, the matrix consists of an exopolysaccharide, which is specified by the *epsA-O* operon, and a secreted protein TasA, which is encoded by the *yqxM-sipW-tasA* operon. Past and present evidence establish that the *epsA-O* and *yqxM-sipW-tasA* operons are controlled by the repressor proteins SinR and AbrB. Here, we report the identification of a novel regulatory protein Slr that promotes transcription of the *yqxM-sipW-tasA* operon but is not needed for expression of the *epsA-O* operon. We further show that the gene for Slr is itself under the negative control of SinR and AbrB. These findings reveal that matrix production is governed by an intricate network involving the interplay of negatively- and positively-acting regulatory proteins.

Introduction

The gram-positive soil bacterium *Bacillus subtilis* is capable of forming surface-attached, multicellular communities or biofilms (Branda *et al.*, 2001; Hamon and Lazazzera, 2001). Some strains of domesticated *B. subtilis* produce thin, floating biofilms at the liquid-air interface (pellicles) and exhibit some measure of colony architecture on solid medium (Branda *et al.*, 2004; Veening *et al.*, 2006). However, robust pellicle formation and complex colony architecture is primarily observed in wild, undomesticated strains. The pellicles and colonies of these wild strains exhibit wrinkles with tongue-like aerial projections that serve as preferential sites for sporulation (Branda *et al.*, 2001).

Biofilms consist of long chains of cells in parallel arrangement (Branda *et al.*, 2001). The bundled chains are held together by an extracellular matrix that is primarily composed of exopolysaccharide and protein (Branda *et al.*, 2006). The 15-gene operon *epsA-O* (henceforth simply *eps*) encodes the biosynthetic machinery required to produce the exopolysaccharide (Branda *et al.*, 2001; Kearns *et al.*, 2005), whereas the protein component is encoded by *tasA*, the terminal gene in the *yqxM-sipW-tasA* operon (henceforth simply *yqxM*) (Branda *et al.*, 2006). The secretion of TasA requires processing of its N-terminal signal sequence by the signal peptidase SipW (Stover and Driks, 1999). Cells lacking both components of the extracellular matrix form flat, featureless colonies and are unable to form pellicles (Branda *et al.*, 2006). Interestingly, only a fraction of the cells in a culture are responsible for producing matrix for the entire community when the *eps* and *yqxM* operons are initially induced (Chai *et al.*, 2008). Moreover, fluorescence microscopy reveals that the biofilm is a dynamic community

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composed of at least three cell types (motile cells, sporulating cells and matrix-producing cells) whose relative proportions change with time (Vlamakis and Aguilar et al., 2008).

Because of the importance of the extracellular matrix to biofilm formation, a central challenge has been to elucidate the regulatory circuitry that governs the expression of the *eps* and *yqxM* operons. These operons are under the negative control of SinR, which binds to and blocks transcription of both operons (Kearns et al., 2005; Chu et al., 2006). Derepression is achieved by the action of the antirepressor SinI, which is produced under the control of Spo0A, a response regulator that also governs entry into sporulation (Bai et al., 1993; Hoch 1993; Shafikhani et al., 2002). An additional regulatory protein implicated in biofilm formation is the repressor AbrB. Using laboratory strains of *B. subtilis*, it was previously shown that the dependence of biofilm formation on Spo0A can be bypassed by a mutation in the gene for AbrB (Hamon and Lazazzera, 2001; Hamon et al., 2004; Veening et al., 2006). This finding suggested that AbrB is a repressor of one or more genes involved in biofilm formation. Indeed, microarray analyses and biochemical experiments identified the *yqxM* operon as a target of AbrB (Hamon et al., 2004; Strauch et al., 2007).

The initial goal of the present work was to further evaluate potential targets of AbrB in biofilm formation and to elucidate the relative contributions of AbrB and SinR to controlling the expression of the matrix operons. During the course of this investigation, we discovered an additional regulatory protein, called Slr, that contributes to the control of matrix production. We show that Slr is needed for the transcription of the *yqxM* operon and that the gene for Slr is itself under the negative control of SinR and AbrB. These findings reveal that biofilm formation is governed by a regulatory circuit involving two parallel pathways of repression mediated by SinR and AbrB and an embedded regulatory loop involving an apparent activator protein Slr (Fig. 1).

Results

AbrB represses the *eps* and *yqxM* operons for matrix production

As a starting point for this work, we took advantage of the capacity of the undomesticated strain 3610 to form robust biofilms on solid medium and in standing liquid medium. Accordingly, we created an insertion/deletion (null) mutation of *abrB* in strain 3610 by replacing the *abrB* coding sequence with a drug-resistance gene. The resulting mutant formed hyper-wrinkled colonies on solid medium and very thick, architecturally complex pellicles (Fig. 2A).

The hyper-wrinkled phenotype of the *abrB* mutant was similar to that of a *sinR* mutant (Kearns et al., 2005). We therefore hypothesized that, like SinR, AbrB represses transcription of the *eps* and *yqxM* operons. Indeed, *sipW*, a member of the *yqxM* operon, was one of two genes previously identified as targets of AbrB and reported to be involved in biofilm formation (Hamon et al., 2004). The other gene so identified, *yoaW*, encodes a protein of unknown function. We created a null mutation of *yoaW* in strain 3610, but in our hands the resulting mutant formed biofilms that were indistinguishable from those of the wild type strain on both solid and in liquid medium (Fig. S1). Therefore, we restricted our analysis to the *eps* and *yqxM* operons.

Next, we measured the effect of the *abrB* mutation on transcription from the promoters for the *eps* and *yqxM* operons using fusions to *lacZ*. The absence of AbrB results in constitutive bundling, which interferes with accurate measurements of cell number. We therefore introduced a mutation in *epsH* (a member of the *eps* operon) to block exopolysaccharide production and hence alleviate bundling (Kearns et al., 2005; Chu et al., 2006). The results of Table 1 show that the absence of AbrB did indeed derepress the *eps* and *yqxM* operons.

Furthermore, a mutation in *abrB* bypassed the requirement for Spo0A for *eps* and *yqxM* operon expression (Table 1).

We wondered whether this derepression was an indirect effect on the level of expression of *sinI*, which encodes an antagonist of the SinR repressor (Bai et al., 1993). SinR is known to bind to the promoter regions for, and inhibit the transcription of, both matrix operons (Kearns et al., 2005; Chu et al., 2006). However, the results shown in Table S1 indicate that the absence of AbrB failed to increase the level of expression of *lacZ* fused to the *sinI* promoter. We also performed immunoblotting assays using antibodies against SinI and failed to observe an increase in SinI levels in the absence of AbrB (data not shown).

AbrB and SinR act independently in repressing the matrix operons

Next, we asked whether AbrB and SinR exert their repressive effects independently. To do so, we created double mutants lacking both repressors. The results of Table 1 show that the contributions of the two repressors was approximately additive. That is, the level of expression of each operon for the double mutant was approximately equal to the sum of the expression levels for the single mutants. Interestingly, the contribution of SinR to the repression of the *eps* operon was substantially greater than the contribution of AbrB. Conversely, the contribution of SinR to the repression of the *yqxM* operon was somewhat less than that for AbrB.

AbrB binds to the promoter region for the *yqxM* operon

To investigate whether AbrB binds to the *eps* and *yqxM* promoter regions, we performed electrophoretic mobility shift assays (EMSA) using purified AbrB. As shown in Fig. 3A, AbrB retarded the electrophoretic mobility of a DNA fragment containing the *yqxM* promoter region (P_{yqxM}) with a similar binding affinity to a known target of AbrB, the *comK* promoter region (Hamoen et al., 2003). However, we were unable to detect any change in the electrophoretic mobility of a DNA fragment containing the *eps* promoter region. This result was similar to that seen using a DNA fragment containing a promoter known to not be under the control of AbrB (*recA*).

DNase I footprinting experiments using radiolabeled P_{yqxM} -containing DNA were employed to map the AbrB binding site in the *yqxM* upstream regulatory region, which was centered at position -133 relative to the transcription start site (Fig. 3B). This region was relatively refractory to DNase I digestion, but we detected two sites of weak but reproducible protection (indicated by the ticks) and a site of pronounced hypersensitivity at position -119. AbrB binding sites in the *yqxM* promoter region were independently examined by Strauch et al. (2007). The length of -133 AbrB binding site detected in the present work was consistent with (although slightly smaller; see dotted box in Fig. 4A) than that mapped by Strauch et al. (2007). Strauch et al. (2007) additionally detected an upstream binding site centered at position -182, which was absent in the P_{yqxM} -containing DNA fragment used in our analysis.

Our results together with those of Strauch et al. (2007) and Hamon et al. (2004) indicate that both operons for matrix production are under the negative control of AbrB. In the case of *eps*, we failed to detect binding of the repressor to the operon's promoter region, and hence we do not know whether this control is direct or indirect. In contrast, the promoter region for the *yqxM* operon does contain binding sites for AbrB.

***yqxM* operon transcription requires an activator**

To test the functional significance of the AbrB binding sites, we created a deletion of the P_{yqxM} regulatory region extending from the upstream direction to position -113 and fused this to a promoterless *lacZ* gene (Fig. 4A). The resulting deletion-mutated fusion retained the -10

and -35 sequence elements of the promoter but not the AbrB binding sites. Rather than observing an increase in transcription, as had been expected, the deletion-mutated construct exhibited a slightly lower level of expression than that of the full-length *lacZ* fusion. Based on this observation, we suspected that the downstream endpoint of the -113 deletion was in or near a sequence that was needed for efficient transcription from the promoter. Indeed, the -10 (TACTCT) and -35 (TTTAAA) sequences for the *yqxM* promoter are poor matches to the canonical sequence elements (TATAAT and TTGACA, respectively) recognized by RNA polymerase containing the house-keeping sigma factor σ^A (Chu et al., 2006; Moran et al., 1982), a frequent characteristic of promoters that are under positive control. Thus, we hypothesized that in addition to being subject to negative regulation by AbrB and SinR, P_{yqxM} is subject to positive regulation from an activator sequence located in the vicinity of the -113 deletion.

To investigate this hypothesis further, we created two additional deletions with downstream endpoints at positions -100 and -64. Strikingly, both deletion-mutated fusions were severely impaired in transcription (Fig. 4A). We infer that the region between -113 and -100 contained a sequence element that was required for transcription from P_{yqxM} .

This possibility was reinforced by the observation that the region in question contained a direct repeat of the sequence TGAGCAA centered at positions -100 and -110. We hypothesized that this direct repeat was the binding site for an unknown activator. We refer to the upstream heptad as the 5' repeat and the downstream heptad as the 3' repeat. To investigate the significance of the repeats, we created nucleotide substitution mutations of each repeat separately and together. We observed a slight decrease in *lacZ* expression levels when either the 5' or the 3' repeat alone was mutated and a dramatic decrease in *lacZ* expression when both were mutated (Fig. 4B). All together, the above results suggest that P_{yqxM} is under the positive control of an activator protein that binds to direct repeat sequences upstream of P_{yqxM} to stimulate transcription.

An attractive candidate for the hypothesized activator protein was Slr for the following reasons. First, Slr is highly similar to SinR, a known, direct (negative) regulator of *yqxM*. Slr contains a putative helix-turn-helix domain in its N-terminal region whose amino acid sequence is 41% identical and 73% similar to that of SinR (Fig. 5A). In fact, SinR is antigenically similar to Slr as antibodies directed against SinR also detected Slr protein in immunoblot assays (Fig. S2). In addition, the C-terminal domain of Slr contains a region of similarity to SinI, the antagonist of SinR (28% identical, 65% similar) (Fig. 5A). Second, the gene encoding *slr* is located adjacent to, and in divergent orientation from, the *eps* operon (Fig. 5B).

The SinR-like protein Slr stimulates transcription from P_{yqxM}

To investigate whether Slr is involved in biofilm formation, we replaced the *slr* coding sequence with a drug-resistance gene and found that the resulting null mutant formed flat, featureless colonies and thin, flat pellicles (Fig. 2A). This mutant could be complemented with a copy of *slr* inserted into the chromosome at the *amyE* locus (Fig. 2A). That an Slr mutant exhibits a defect in biofilm formation was independently noted by Kobayashi (2007) in a large-scale survey of the effect of transcriptional regulator mutants on pellicle formation.

Next, we investigated whether Slr is needed for transcription from the promoters for *eps* or *yqxM* using *lacZ* fusions to P_{eps} and P_{yqxM} . Interestingly, expression of P_{eps} -*lacZ* exhibited little or no dependence on Slr, whereas expression of P_{yqxM} -*lacZ* was markedly impaired in the absence of the putative regulatory protein (Fig. 6). Whereas SinR is a repressor, its homolog Slr acts to stimulate transcription in a manner that is selective for *yqxM* and not *eps*.

We next addressed the question of whether *yqxM* is the only operon required for biofilm formation that depends on Slr for its transcription. To do so, we overexpressed the *yqxM* operon

using an IPTG-inducible promoter in an *slr* mutant background. As shown in Fig. 2B, the induction of the *yqxM* operon largely restored complex colony architecture and pellicle formation to the *slr* mutant. Thus, the *yqxM* operon is the major, if not the only, target of the Slr activator required for biofilm formation.

Interestingly, the dependence both of *yqxM* transcription and of biofilm formation on Slr could be partially bypassed by the absence of SinR but not by the absence of AbrB (Fig. S3). That is, a *sinR* mutation (but not an *abrB* mutation) was partially epistatic to the effects of an *slr* mutation. We infer from this that P_{yqxM} is able to function to a significant extent when Slr is absent under circumstances when it is entirely freed of the repressive effects of SinR.

A simple interpretation of the results so far is that Slr binds to, and stimulates transcription from, P_{yqxM} . In an effort to test this idea, we attempted to perform EMSA experiments using purified Slr protein. However, the full-length Slr protein was insoluble and efforts to solubilize and renature the protein failed. We were able to obtain soluble versions of Slr using only the N-terminal DNA-binding portion of Slr as well as a GST-tagged version of full-length Slr. We performed EMSA experiments using these two versions of Slr and found that both proteins bound DNA in a non-specific manner (Fig. S4 and data not shown). Thus, Slr is indeed a DNA-binding protein but we were unable to determine whether it acts directly on the P_{yqxM} promoter region.

If Slr does act directly on P_{yqxM} , it is conceivable that it does so by binding to the above identified heptad repeat. If so, it is pleasing to note that the heptad sequences are absent from the regulatory region of *eps*, whose transcription, as we have seen, is not dependent on Slr. On the other hand, mutations of both of the 5' and 3' heptad sequences more severely impaired *yqxM* transcription (Fig. 4B) than did the absence of Slr (Fig. 6). It is conceivable, therefore, that an additional unknown regulatory protein also binds to the heptad sequences or that the heptad sequences additionally act in *cis* to promote transcription.

slr is under the negative control of SinR and AbrB

Previously, we mapped SinR binding sites in the intergenic region between the divergently oriented *eps* operon and the *slr* gene (Kearns et al., 2004). We found that SinR bound to two regions: a pair of inverted repeat sequences located proximal to *eps* and three direct repeat sequences located just upstream of *slr* (Fig 5B). The direct repeats are centered at positions -31, -39, and -60 upstream from the *slr* open reading frame (Fig. 5B). We wondered whether these SinR binding sites were responsible for repressing the transcription of the *slr* gene. To test this hypothesis, we fused the *slr* promoter to a promoterless *lacZ* gene and monitored *lacZ* expression in a *sinR* mutant. Again, a mutation in the *epsH* gene was introduced to relieve constitutive cell bundling. The *epsH* mutation had little or no effect on the transcription from the *slr* promoter (Table 2). In the absence of SinR, we observed a marked increase in *lacZ* expression, suggesting that SinR is a repressor of *slr* (Table 2). In confirmation of this, we observed a decided increase in Slr levels in the absence of SinR (Fig. S2).

AbrB and SinR both repress at least two operons, *eps* and *yqxM*, required for biofilm formation. Therefore, we wondered if AbrB, like SinR, also represses the transcription of *slr*. As shown in Table 2, the absence of AbrB resulted in the derepression of *slr*. The extent of derepression was, however, much less than that caused by the absence of SinR, and the level of expression of *slr* in a *sinR* *abrB* double mutant was no higher (indeed somewhat lower) than in a *sinR* single mutant. We therefore conclude both SinR and AbrB contribute to the control of *slr* but that SinR plays a larger role in repressing *slr* than does AbrB. All together, we have shown that SinR, directly, and AbrB, directly or indirectly, repress at least three transcription units required for biofilm formation (Fig. 1).

Discussion

The present investigation has revealed important new features of the regulatory circuitry governing the expression of the *eps* operon, which specifies exopolysaccharide production, and the *yqxM* operon, which encodes the matrix protein TasA. The most important contribution is the discovery of an additional regulatory protein, Slr, involved in matrix production. In addition, we have identified additional targets of the repressor AbrB, which was previously known to be involved in biofilm formation (Hamon et al., 2004). Finally, we show that AbrB and SinR act in parallel to control the expression of the *eps* and *yqxM* operons and that all three regulators, Slr, AbrB, and SinR, converge in governing the production of the matrix protein TasA (Fig. 1).

We were alerted to the possibility that Slr is involved in biofilm formation by the following considerations. First, the gene for Slr is located immediately adjacent to, and shares a common regulatory region with, the *eps* operon. Second, Slr is strikingly similar in amino acid sequence to SinR, which, as we have argued, is largely dedicated to the expression of genes and operons involved in biofilm formation (Kearns et al., 2005). Thus, the N-terminal region of Slr is homologous to the helix-turn-helix in the N-terminal, DNA-binding region of SinR. Also, the C-terminal region of Slr resembles SinI, the antirepressor for SinR (Bai et al., 1993). Finally, our dissection of the regulatory region for the *yqxM* operon revealed an apparent requirement for an unknown activator.

Indeed, our present results, and the independent finding of Kobayashi (2007) reveal that Slr is required for biofilm formation. In addition, we have shown that the *slr* gene is under the negative control of SinR and that it is required for induction of the *yqxM* operon. Interestingly, and even though *slr* is located adjacent to *eps*, it is not required for the expression of the exopolysaccharide operon. In fact, our results suggest that *yqxM* is the principal, if not the exclusive, target of Slr's action in promoting biofilm formation. We were unable to settle the issue of whether Slr acts directly on *yqxM*, but the simplest interpretation of our results is that it activates transcription by binding to the promoter. If so, it is conceivable that its target is the 5' and 3' heptad repeat sequences, although with the important caveat that mutation of the heptads impaired transcription more severely than did the absence of Slr. Finally, and reinforcing the view that both SinR and Slr are largely dedicated to the control of genes involved in biofilm formation, it is pleasing to note that *sinR* and *slr* each flank one of the two operons governing the production of extracellular matrix.

We have also integrated AbrB into the circuitry that governs biofilm formation. We have shown that AbrB represses both the *eps* operon and the *slr* gene, although we do not know whether this effect is direct or indirect. In addition, our present findings together with those of two other groups indicate that AbrB represses the *yqxM* operon (Hamon et al., 2004; Strauch et al., 2007). Furthermore, our results and those of Strauch et al., (2007) indicate that AbrB acts directly on *yqxM*. Finally, we have shown that AbrB and SinR act independently in repressing their targets. That is, the effects of the absence of both repressors was not significantly greater than the sum of the effects of the absence of either AbrB or SinR alone.

In toto, these findings lead to the view that SinR and AbrB represent parallel pathways, both controlled by the response regulator, Spo0A. Thus, activation of Spo0A sets in motion events (induction of the antirepressor gene *sinI* and repression of *abrB*) that relieve AbrB- and SinR-mediated repression. The ultimate targets of this repression are the *eps* and *yqxM* operons, which govern extracellular matrix production and are the only genes and operons directly involved in formation of the extracellular matrix so far identified.

This notion that Spo0A sets in motion two parallel pathways might at first glance appear to be at odds with epistasis experiments involving the *abrB* gene. Thus, an *arbB* mutation is known

to be epistatic (Hamon and Lazazzera, 2001) to a *spo0A* mutation but not to a *sinI* mutation (Kearns et al., 2005). That is, an *abrB spo0A* double mutant makes hyper-wrinkled colonies whereas an *abrB sinI* double mutant makes smooth colonies. Yet, *spo0A* is required for the expression of *sinI* (Shafikhani et al., 2002; Fujita et al., 2005). Therefore, in the *abrB spo0A* double mutant, the *sinI* gene should be off and hence an *abrB spo0A* double mutant should be equivalent to **an** *abrB sinI* double mutant. How are we to resolve this paradox? We propose that *sinI* is not totally silent in the absence of Spo0A. Rather, it is transcribed at a low but significant level by read-through transcription from the upstream operon (*yqhH-yqhG*). In keeping with this hypothesis, we note that the intergenic region between the upstream operon and *sinI* does not contain a conspicuous transcriptional terminator, and a drug-resistance cassette inserted in this region (with its promoter oriented away from the *sinI sinR* operon) impairs biofilm formation (Y. Chai, unpublished results). We propose that read-through transcription from the upstream operon augments transcription of *sinI* and that in a *spo0A abrB* double mutant, a small amount of SinI is produced, which suffices to relieve SinR-mediated repression partially. We propose that when partially inhibited by a low level of SinI, SinR is unable to silence the *eps* and *yqxM* operons without the additional contribution of AbrB.

In summary, the present work reveals that the circuitry for extracellular matrix production is unexpectedly intricate. It involves four transcriptional regulatory proteins (Spo0A, SinR, AbrB and Slr), two of which (SinR and Slr) are largely, if not exclusively, dedicated to the control of the two operons involved in matrix production. In addition, each operon is the direct or indirect target of more than one regulatory protein, two in the case of *eps* and three in the case of *yqxM*. We infer that this high level of regulation serves to ensure tight regulation of the operons, such that the matrix is produced only under the appropriate circumstances.

Materials and Methods

Strains and growth conditions

Strain 3610 and its derivatives were grown at 37°C in Luria-Bertani broth (LB; 10 g tryptone, 5 g yeast extract, and 5 g NaCl per L) or on solid medium containing LB supplemented with 1.5% agar. For formation of architecturally complex colonies, cells were toothpick-inoculated onto MSgg medium (Branda *et al.*, 2001) containing 1.5% Bacto agar and incubated at 22°C for 72 hours. For pellicle formation, 10 µl of mid-log phase culture was inoculated into 10 ml minimal MSgg medium and incubated statically at 22°C for 72 hours. When appropriate, antibiotics and supplements were included at the following concentrations: 100 µg ml⁻¹ X-gal, 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 linomycin.

Strain construction

All insertion/deletion mutations were generated using long flanking homology PCR using primers indicated in Table S3. The *spo0A::spec* and *epsH::tet* constructs were previously generated (Fawcett et al., 2000; Kearns et al., 2005). The *abrB::kan* construct was a gift from E. Hobbs. The strains were transformed into competent PY79 cells and mutations were transferred into the 3610 background using SPP1-mediated generalized transduction (Yasbin and Young, 1974; Kearns et al., 2004). All strains used in this study are listed in Table S2.

Complementation and reporter expression constructs

All primers used in the construction of plasmids are listed in Table S3. To generate the *Pslr-lacZ* reporter construct, a PCR product containing the *Pslr* promoter was amplified from 3610 chromosomal DNA using primers Pslr-F1 and Pslr-R1. The PCR product was cloned into the *EcoRI* and *BamHI* sites of plasmid pDG268 (Antoniewski et al., 1990). To generate the *PsinI-lacZ* reporter construct, a PCR product containing the *PsinI* promoter was amplified from

3610 chromosomal DNA using primers -499sinI and -2sinI. The resulting PCR product was cloned into the *EcoRI* and *BamHI* sites of pDG268.

To generate the deletion constructs of P_{yqxM} -*lacZ*, PCR products containing various versions P_{yqxM} were amplified from 3610 chromosomal DNA using the primer -26yqxM in combination with the following forward primers: for the -113 construct, -175yqxM; for the -100 construct, -160yqxM; and for -64 construct, -126yqxM. The PCR products were then cloned into the *EcoRI* and *BamHI* sites of plasmid pDG268. To generate the mutation of the 3' site of the heptad repeats, two PCR products were amplified from the primers -302yqxM/ALM3 and -26yqxM/ALM1. The two PCR products were ligated together using *XhoI* sites and cloned into the *EcoRI* and *BamHI* sites of pDG268. To generate the mutation of the 5' site of the heptad repeats two PCR products were amplified from the primers -302yqxM/ALM5 and -26yqxM/ALM4. The two PCR products were ligated together using *XhoI* sites and cloned into the *EcoRI* and *BamHI* sites of pDG268. To generate the mutations in both sites of the heptad repeats two PCR products were amplified from the primers -302yqxM/ALM2 and -26yqxM/ALM1. The two PCR products were ligated together using *XhoI* sites and cloned into the *EcoRI* and *BamHI* sites of pDG268.

To generate the *slr* complementation construct, a PCR product containing the promoter and open reading frame of *slr* was amplified using the primers slrcomp_F and slrcomp_R. The PCR product was cloned into *EcoRI* and *BamHI* sites of plasmid pDG364 (Karmazyn-Campelli et al., 1992).

β-galactosidase assay

Cells were harvested from shaking cultures growing at 30°C in MSgg broth. Cells were collected in 1 ml aliquots and suspended in an equal volume of Z buffer (40 mM NaH₂PO₄, 60 mM Na₂HPO₄, 1 mM MgSO₄, 10 mM KCl, and 38 mM 2-mercaptoethanol). Lysozyme was added to each sample to a final concentration of 0.2 mg ml⁻¹ and incubated at 30°C for 15 minutes. Each sample was diluted in Z-buffer to a final volume of 1 ml and the reaction was started with 200 μl of 4 mg ml⁻¹ 2-nitrophenyl β-D-galactopyranoside in Z buffer and stopped with 500 μl 1 M Na₂CO₃. After centrifuging the samples for 20 minutes at 15 000 × g, the OD₄₂₀ of the reaction mixture was measured. The β-galactosidase specific activity was calculated according to the equation: [OD₄₂₀/(time × OD₆₀₀)] × dilution factor × 1000.

Protein expression constructs

To generate plasmids for the expression of N-terminal 6-histidine translation fusions to AbrB and the N-terminus of Slr (pEH213 and pFC588, respectively), PCR products were generated using the primers ECH318/ECH336 and +1slr/+339slr, respectively. The PCR products were cloned into the *NdeI* and *XhoI* sites of plasmids pET15b (for AbrB) and pET21b (for Slr) (Novagen). The plasmids were then transformed into *Escherichia coli* BL21(DE3) RIL-CodonPlus cells (Stratagene).

Protein purification

E. coli strains were grown at 30°C in 1 L of LB supplemented with 100 μl ml⁻¹ ampicillin and 50 μg ml⁻¹ chloramphenicol until OD₆₀₀ reached ~1.0. IPTG was added to a final concentration of 1 mM and the culture grew for two additional hours at 30°C. The cells were harvested, resuspended in 30 ml of lysis/binding buffer [20 mM Tris-HCl (pH8.0), 20% (v/v) glycerol, 100 mM NaCl, and 20 mM imidazole], and lysed by sonication. The lysate was centrifuged at 32,000 × g and the resulting supernatant was loaded onto a column containing 1 ml of Ni²⁺-NTA agarose beads (Qiagen) that had been equilibrated in lysis/binding buffer. The beads were washed five times with 2 ml aliquots of lysis/binding buffer. A stepwise elution was performed with 2 ml aliquots of buffer [20 mM Tris-HCl (pH8.0), 20% (v/v) glycerol, 100 mM NaCl]

supplemented with 50 mM, 100 mM, 200 mM, and 400 mM imidazole. The fractions containing purified protein were pooled and dialyzed against 4 L dialysis buffer [20 mM Tris-HCl (pH8.0), 50% (v/v) glycerol, 100 mM NaCl, 1mM EDTA, 1mM DTT] overnight at 4°C. The dialyzed protein was stored at -20°C for AbrB and -80°C for Slr.

Electrophoretic mobility shift assay (EMSA)

Radiolabeled probes were generated by PCR using 3610 chromosomal DNA and the following primer combinations: -246epsA/+4epsA (P_{eps}), ECH341/ECH342 (P_{recA}), -270yqxM/+14yqxM (P_{yqxM}), and ECH343/ECH344 (P_{comK}). The *gfp* probe was generated by PCR using plasmid pKL147 DNA as a template and primers JSK362/JSK445. Each probe was PCR purified and 5' end labeled with 10 μ Ci of [γ -³²P]-ATP (NEG002A, New England Nuclear) using polynucleotide kinase (New England Biolabs). Various concentrations of purified AbrB or N-Slr were added to approximately 100 nM of radiolabeled probe (Kearns *et al.*, 2004). N-Slr-DNA binding reactions were carried out in 30 μ l volumes, including binding buffer (10 mM Tris HCl, 50 mM NaCl, 1mM EDTA, 5% glycerol, 1mM dithiothreitol (DTT), 10 μ g ml⁻¹ bovine serum albumin) and 25 μ g ml⁻¹ polydeoxyinosinic-deoxycytidylic acid (poly dI-dC), at room temperature for 20 minutes. A 6% polyacrylamide 0.5 \times TBE gel was loaded with 10 μ l of each binding reaction and resolved for 1 hour at 100 mV. AbrB-DNA binding reactions were carried out in 30 μ l volumes including binding buffer [20mM Tris-HCl (pH8.0), 100 mM KCl, 5mM MgCl₂, 0.5 mM DTT, 10% glycerol, 0.05% Nonidet P40, 0.05 μ g ml⁻¹ bovine serum albumin] containing 25 μ g ml⁻¹ poly dI-dC, at 37°C for 20 minutes. A 6% polyacrylamide 1 \times TAE gel was loaded with 10 μ l of each binding reaction and resolved for 2.5 hours at 50 mV.

DNase I footprinting assay

Primer -21yqxM_R was 5' end labeled with [γ -³²P]-ATP and polynucleotide kinase. A PCR product of the P_{yqxM} region was generated using 3610 chromosomal DNA as a template and 50 pmol of labeled primer -21yqxM_R and unlabeled primer -244yqxM_F. The PCR product was then PCR purified and the c.p.m. was measured using a scintillation counter. Approximately 30 000 c.p.m. of radiolabeled PCR product was added to varying concentrations of AbrB protein in 100 μ l 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 BSA) with 5 μ g ml⁻¹ poly dI-dC and incubated at room temperature for 20 minutes. Each sample was then incubated for 30 seconds with 2 μ l DNaseI (1:25 dilution of 1 U μ l⁻¹ stock, Invitrogen) before digestion was stopped by adding 25 μ l of stop solution (1.5 M sodium acetate pH 5.3, 20 mM EDTA, and 400 μ g ml⁻¹ glycogen). The reactions were then ethanol precipitated and resuspended in 8 μ l of formamide loading dye (80% deionized formamide, 10 mM EDTA, 1 mg ml⁻¹ xylene cyanol, and 1 mg ml⁻¹ bromophenol blue). A total of 6 μ l of each reaction was loaded on an 8% sequencing gel and resolved before being visualized using phosphoimaging.

Western blot assay

Cells of each strain were grown to 1 OD₆₀₀ in LB broth at 37°C, harvested, resuspended to 11.5 OD₆₀₀, and lysed for 30 minutes in lysis buffer (20 mM Tris pH 7.0, 10 mM EDTA, 10mg/ml lysozyme, 1 mg/ml DNase, and 10 mg/ml RNase A). An amount of 6X sample buffer (0.5 M Tris pH 6.8, 15% glycerol, 0.1g/ml SDS, 1.2 mg/ml bromophenol blue, 60 μ l/ml β -mercaptoethanol) was added to the cell suspension to a final concentration of 1X and the samples were boiled for 1 minute. 10 μ l of each sample (0.1 OD₆₀₀ equivalent) was loaded per lane and proteins were resolved by SDS-polyacrylamide gel electrophoresis (15%). Proteins were transferred onto nitrocellulose membrane by electroblotting for 1 hour at 400 mA. The membrane was blocked for 1 hour at room temperature in TBSTM buffer (20 mM Tris pH 8.0, 200 mM NaCl, 0.1% Tween 20, 5% instant non-fat dry milk). The membrane was probed with a 1:20,000 dilution of anti-SinR primary antibody in TBSTM buffer overnight at 4°C. The

membrane was washed three times in TBSTM and probed with a 1:10,000 HRP conjugated goat anti-rabbit secondary antibody in TBSTM buffer for one hour at room temperature. The blot was developed with an ECL chemiluminescence detection kit (Biorad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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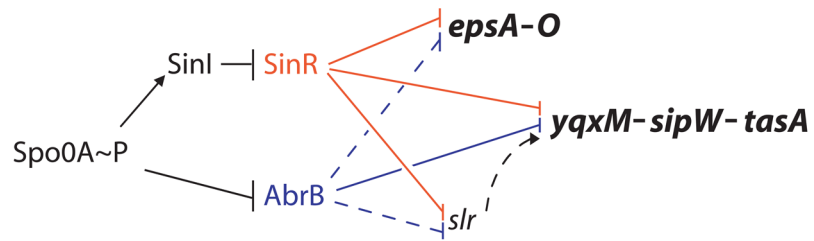


Figure 1. Regulatory circuit governing biofilm formation

Arrows indicate activation of transcription and T bars indicate repression. Solid lines denote direct regulation and the dashed lines denote cases in which it is unknown if the regulation is direct. Red indicates SinR and its targets and blue denotes AbrB and its targets.

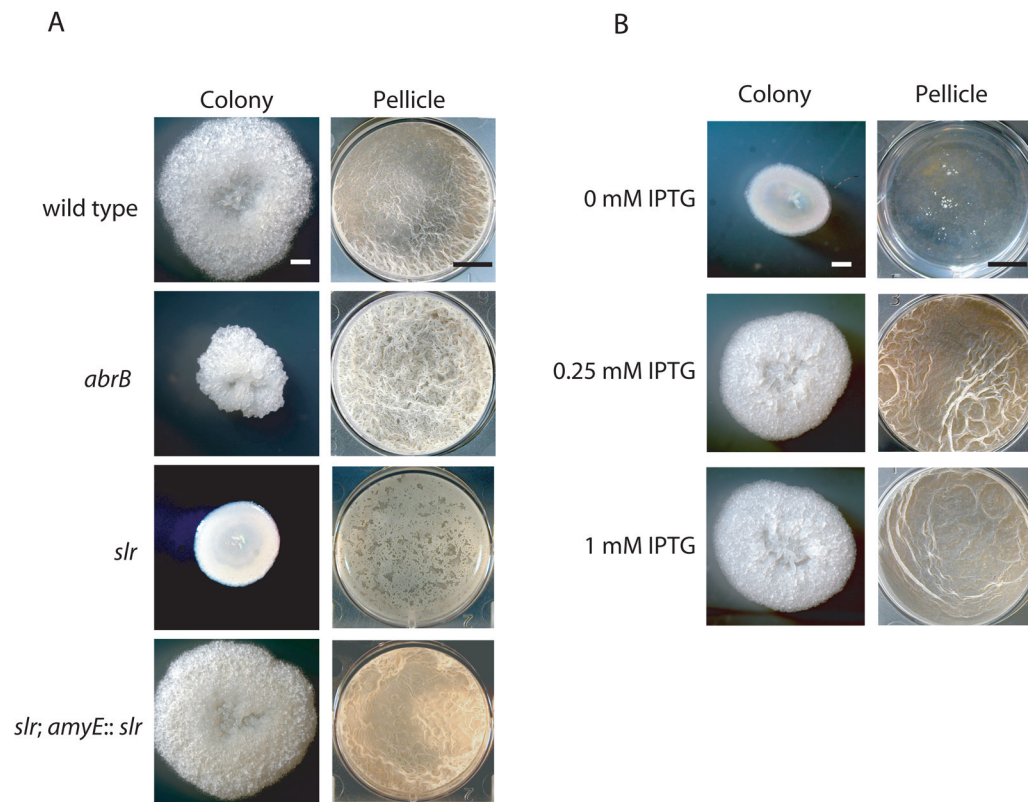


Figure 2. Effect of regulatory mutations on biofilm formation

'Colony' column depicts $16\times$ images of colonies grown on MSgg semi-solid agar medium for 3 days at 22°C . Scale bar is 1 mm. 'Pellicle' column shows top-down images of 6-well microtitre plates in which cells have been grown statically in MSgg medium for 3 days at 22°C . Scale bar is 1 cm. A) Opposing phenotypes of *abrB* and *slr* mutations. The indicated wild type and mutant strains were as follows: wild type (3610), *abrB* (FC280), *slr* (FC525), *slr; amyE::slr* (FC536). B) The *slr* mutant phenotype is rescued by *yqxM* operon overexpression. IPTG was added to MSgg medium at the indicated concentrations. The strain (FC600) was mutant for *slr* and contained $yqxM\Omega_{\text{IPTG}}\text{-}yqxM\text{-}sipW\text{-}tasA$.

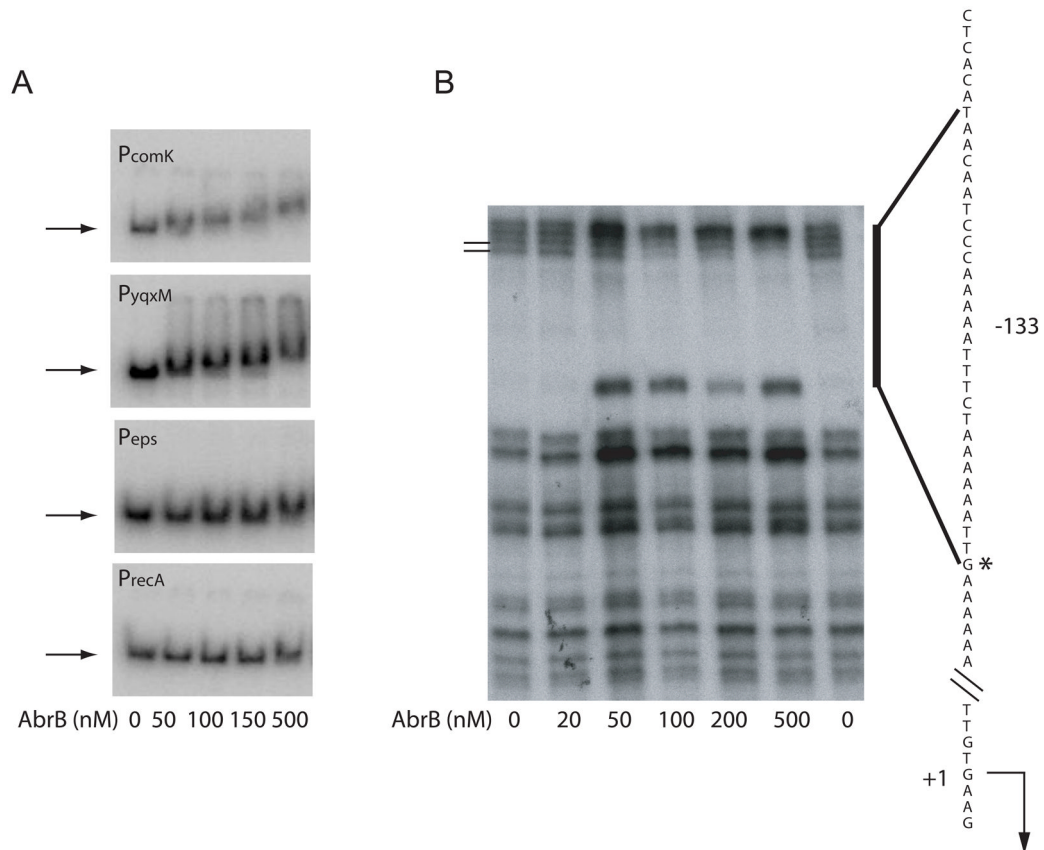


Figure 3. AbrB binds to the *yqxM* but not to the *eps* promoter

A) EMSA in which radiolabeled DNA's (denoted in the upper left corner of each panel) were mixed with purified His₆-AbrB at the indicated concentrations. The sizes of the DNA fragments containing P_{comK}, P_{yqxM}, P_{epsA}, and P_{recA} were 475, 284, 250, and 435 base pairs, respectively. The horizontal arrow indicates the position of free probe. B) Footprinting experiment in which a 223 bp fragment of DNA containing P_{yqxM} was 5' end labeled at the terminus downstream of the promoter, mixed with purified AbrB at the indicated concentrations, and subjected to treatment with DNase I. The vertical bar indicates the region of AbrB protection. Ticks to the left of the panel indicate the AbrB-protected bands. The corresponding DNA sequence is shown to the right of the panel and was determined from a DNA sequencing ladder. The asterisk corresponds to the site of hypersensitivity. A bent arrow indicates the *yqxM* operon transcription start site. The number to the left of the sequence indicates the *yqxM* transcription start site. The number to the right of the sequence indicates the position of the AbrB binding site relative to the transcription start site.

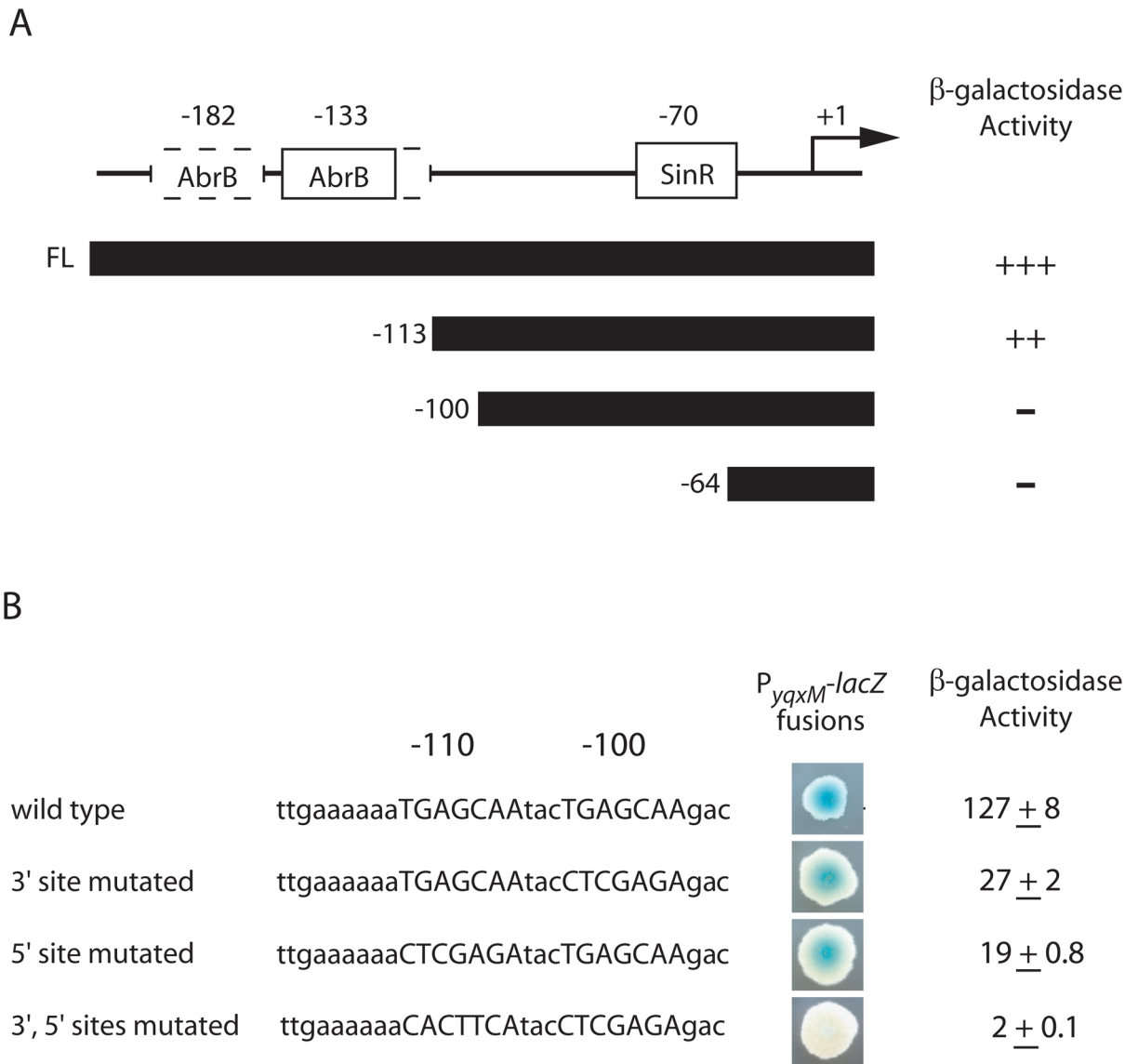


Figure 4. Transcription of *yqxM* requires an upstream, direct repeat sequence

A) The horizontal line depicts the upstream regulatory region of P_{yqxM} . Open boxes denote the indicated repressor binding sites. Dashed lines indicate the *AbrB* binding sites mapped by Strauch et al. (2007). The bent arrow denotes the transcription start site. Numbers above the horizontal line indicate the positions of the repressor binding sites relative to the transcription start site. Black bars represent the region of P_{yqxM} included in each construct. Numbers to the left of each black bar denote the 5' end point of the respective construct. 'FL' indicates the full-length P_{yqxM} reporter construct. The relative level of β -galactosidase activity for each *lacZ* reporter construct is indicated at the right. The '+' symbol denotes high levels of β -galactosidase activity and the '-' symbol indicates background levels of β -galactosidase activity. The following *lacZ* reporter strains were used: full-length (FC591), -112 (FC488), -100 (ALM16), and -64 (FC495). B) Shown is the sequence of the wild type and mutant versions of the direct repeat in the upstream regulatory region of P_{yqxM} . Upper case letters are the sequence of the wild type or mutant versions of the direct repeat. Lower case letters are the surrounding sequence. Numbers above the sequence indicate the positions of each site relative

to the *yqxM* transcription start site. Colonies to the right were grown on MSgg semi-solid agar supplemented with X-gal to detect β -galactosidase activity. The following *lacZ* reporter strains were used: wild type (FC591), 3' mutated (ALM36), 5' mutated (ALM35), 3', 5' sites mutated (ALM37).

A

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Slr 1 MIGRIIRLYRKRKGYSLNQLAVESGVSKSYLSKIERGVHTNPSVQFLKKVSATLEVELTELFD AETMMYEKISGGEEEWRVHLVQAVQAGMEKEELFTFTNRLKKEQPETAS 112
SinR 1 MIGQRIKQYRKEKGYSLSELAEKAGVAKSYLSIERNLQTNPSIQFLEKVSAVLDVSVHTLLD-EKHET EYDGGQLDSEWEKLV RDAMTSVSKKQRFREFLDYQKWRKSQKEE 111
    ***: *: **.****:.*: **:*:***.***.:***:***:***:*.: *:* * . * . :.*. : :*: :*: :*: : * : * :
Slr 113 YRNKLTESNIE-EWKALMAEAREIGLSVHEVKSFLTKGR 152
SinI 1 MKNAQEHFELDQEWVELMVEAKEANISPEEIRKYL LNKSAHPGPAARSHTVNP 57
    :* * . : : * **.***:* . :* :* : : :
    
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B

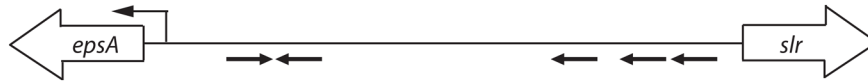


Figure 5. The *slr* gene encodes a SinR-like protein and shares a common regulatory region with the *eps* operon

A) The amino acid sequence alignment of Slr and SinR and SinI. Asterisks indicate identical amino acids and the dots indicate similar amino acids. Numbers indicate the amino acid position. Sequence alignment information was obtained from <http://www.ebi.ac.uk/Tools/clustalw/>. B) A schematic of the shared upstream regulatory region between the *slr* and *epsA* coding regions. Open arrows indicate the open reading frames. Black, horizontal arrows indicate the mapped SinR binding sites. The bent arrow denotes the *eps* transcription start site. The three direct repeats of SinR binding sites on the right are centered at 31, 39, and 60 base pairs upstream from the *slr* coding region.

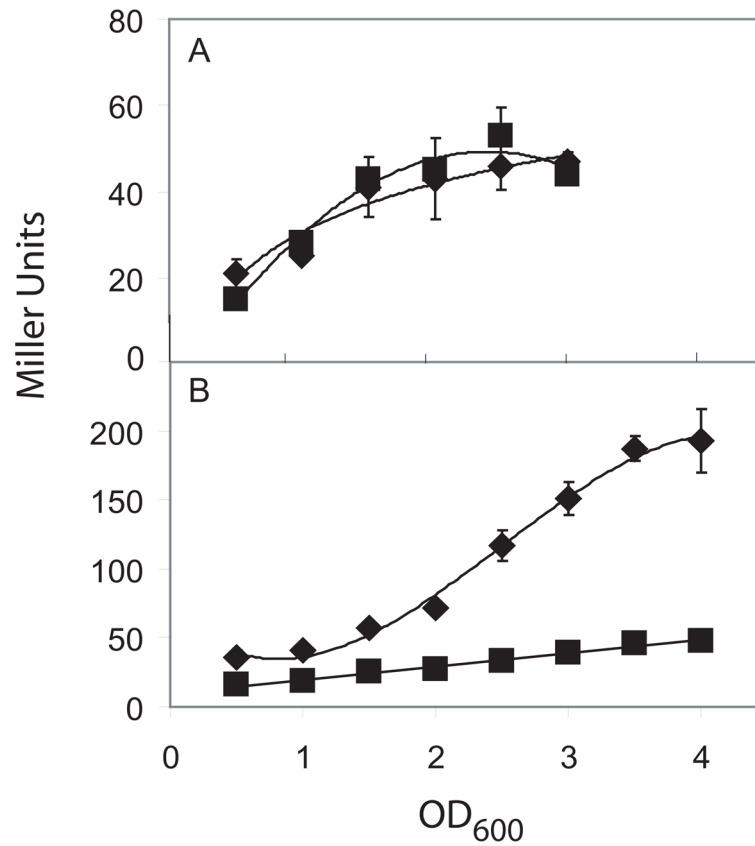


Figure 6. *Slr* is required for transcription of *yqxM* but not *eps*

The indicated strains were grown at 30°C in MSgg and β-galactosidase was measured in Miller Units. A) β-galactosidase expression of P_{eps}-lacZ in wild type (◆)(FC13) and *slr* (■) (FC526). B) β-galactosidase expression of P_{yqxM}-lacZ in wild type (◆) (FC591) and *slr* (■) (FC593).

Table 1AbrB represses the *yqxM* and *eps* operons

Genotype ^a	Activity (MU) ^b	
	P _{<i>eps</i>} - <i>lacZ</i>	P _{<i>yqxM</i>} - <i>lacZ</i>
Wild type	40 ± 1	130 ± 9
<i>spo0A</i>	2.8 ± 0.2	4 ± 0.8
<i>abrB</i>	150 ± 2	1100 ± 31
<i>sinR</i>	1100 ± 13	670 ± 47
<i>spo0A abrB</i>	150 ± 10	1000 ± 28
<i>sinR abrB</i>	1300 ± 20	1600 ± 32

^aThe following P_{*eps*}-*lacZ* containing strains were used FC13, FC413, FC415, FC14, FC444, and FC480. The following P_{*yqxM*}-*lacZ* containing strains were used FC134, FC414, FC416, FC135, FC445, and FC481.

^bβ-Galactosidase activity, calculated in Miller Units (MU), was measured from late-exponential phase cells grown in MSgg and is the average of at least three replicates.

Table 2AbrB and SinR repress *slr*

Genotype ^a	P _{<i>slr</i>} - <i>lacZ</i> activity(MU) ^b
Wild type	7 ± 0.3
<i>epsH</i>	7 ± 0.2
<i>sinR epsH</i>	180 ± 2
<i>abrB epsH</i>	16 ± 1
<i>abrB sinR epsH</i>	133 ± 7

^aThe following P_{*slr*}-*lacZ* containing strains were used FC518, FC519, FC520, FC522, and FC606.

^bβ-Galactosidase activity, calculated in Miller Units (MU), was measured from late-exponential phase cells grown in MSgg and is the average of at least three replicates.