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Bistability and biofilm formation in *Bacillus subtilis*

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Summary

Biofilms of *Bacillus subtilis* consist of long chains of cells that are held together in bundles by an extracellular matrix of exopolysaccharide and the protein TasA. The exopolysaccharide is produced by enzymes encoded by the *epsA-O* operon and the gene encoding TasA is located in the *yqxM-sipW-tasA* operon. Both operons are under the control of the repressor SinR. Derepression is mediated by the antirepressor SinI, which binds to SinR with a 1:1 stoichiometry. Paradoxically, in medium promoting derepression of the matrix operons, the overall concentration of SinR in the culture greatly exceeded that of SinI. We show that under biofilm-promoting conditions *sinI*, which is under the control of the response regulator Spo0A, was expressed only in a small subpopulation of cells, whereas *sinR* was expressed in almost all cells. Activation of Spo0A is known to be subject to a bistable switch, and we infer that SinI reaches levels sufficient to trigger matrix production only in the subpopulation of cells in which Spo0A is active. Additionally, evidence suggests that *sinI* is expressed at intermediate, but not low or high, levels of Spo0A activity, which may explain why certain nutritional conditions are more effective in promoting biofilm formation than others.

Introduction

Most bacteria are capable of forming surface-associated, architecturally complex communities of cells, which are known as biofilms (O'Toole and Kaplan, 2000; Stoodley *et al*., 2002; Kolter and Greenberg, 2006). Biofilms assemble on solid surfaces or as pellicles at air/liquid interfaces. A hallmark of biofilms is the presence of an extracellular matrix that holds the cells together (Branda *et al*., 2005). The matrix typically consists of exopolysaccharides and proteins and sometimes nucleic acid (Sutherland, 2001; Whitchurch *et al*., 2002). Remarkably, the mechanisms governing the production of the matrix differ markedly from bacterium to bacterium, an observation that suggests that the capacity to assemble into communities arose independently many times in the microbial world (Davies *et al*., 1998; Branda *et al*., 2005). Here we are concerned with the mechanisms governing the production of the extracellular matrix in the spore-forming bacterium *Bacillus subtilis*.

Wild strains of *B. subtilis*, such as the 3610 strain used in the present study, form elaborate biofilms in which spore formation takes place preferentially at the tips of aerial structures that protrude from the surface of the community (Branda *et al*., 2001). Biofilm formation and sporulation are also connected in that both processes are dependent on Spo0A, the master regulator for entry into sporulation (Sonenshein, 2000; Branda *et al*., 2001; Hamon and Lazazzera, 2001). Indeed, and as we will argue here, expression of genes involved in matrix production may be a prelude to the process of spore formation. In *B. subtilis*, the matrix consists of an exopolysaccharide, which is produced under the direction of the 15-gene operon *epsA-O* (henceforth simply *eps*) (Branda *et al*., 2001) and the secreted protein TasA, which is encoded within the *yqxM-sipW-tasA* operon (henceforth simply *yqxM*) (Branda *et al*., 2006). Both operons are under the direct negative control of the repressor SinR (Kearns *et al*., 2005; Chu *et al*., 2006). Derepression under conditions promoting biofilm formation occurs, at least in part, through the action of SinI, an antagonist of SinR (Bai *et al*., 1993; Kearns *et al*., 2005). SinI binds to the SinR repressor in a one-to-one stoichiometry, thereby preventing the repressor from binding to DNA (Lewis *et al*., 1996; 1998).

The starting point for this investigation was the puzzling observation that the level of expression of the *sinR* gene, and as a consequence, the level of accumulation of its product, is much higher than that of *sinI*. If SinI acts at a one-to-one stoichiometry with SinR, then how is derepression of the *eps* and *yqxM* operons achieved when the antirepressor is present at much lower concentrations than the repressor? Here we show that *sinI* is expressed at a high level but only in a small subpopulation of the cells, leading to the hypothesis that in these *sinI*-expressing cells the antirepressor reaches a threshold concentration sufficient to trigger depression. We further

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suggest that at the early stages of biofilm formation a subpopulation of *eps*- and *yqxM*-expressing cells supplies matrix to the entire community.

Results

Strategy

The goal of this work was to study the role of the SinR-antagonist, SinI, in the derepression of the operons (*eps* and *yqxM*) governing extracellular matrix formation. A complication in attempting to do so was that biofilms are an architecturally complex community of tightly packed cells in which it is difficult to carry out quantitative studies on gene expression. To circumvent this problem, we took advantage of the fact that transcription from the promoters for the *eps* and *yqxM* operons is strongly induced by 1 h after the end of exponential phase growth under conditions in which the cells are uniformly dispersed in shaking cultures in the biofilm-promoting medium MSgg (Kearns *et al*., 2005; Branda *et al*., 2006; Chu *et al*., 2006). Accordingly, and for the purposes of studying the role of SinI in derepression of the *eps* and *yqxM* operons, we carried out our experiments with cells in homogeneous suspension in shaking cultures.

SinR is much more abundant than SinI

Based on the 1:1 stoichiometry of SinR and SinI in the heteromeric complex of the two proteins (Lewis *et al*., 1996; 1998), the cellular concentration of SinI is expected to be at least as great as that of SinR under conditions in which SinR-controlled genes are derepressed. Indeed, previous Electrophoretic Mobility Shift Assays have shown that the concentration of SinI must be equal to, or in excess of, that of SinR to displace the repressor from its operator (Bai *et al*., 1993; Kearns *et al*., 2005). We were therefore puzzled to discover that the level of expression of the *sinI* gene, as judged by using *lacZ* fused to the *sinI* promoter (P_{sinI}–*lacZ*), was approximately 15-fold lower than that of P_{sinR}–*lacZ* in cells of the wild strain 3610 growing in a medium (MSgg) that promotes biofilm formation (Branda *et al*., 2001) (Fig. 1A).

We wondered whether this difference in promoter activity was reflected in the relative cellular concentrations of the two proteins. Accordingly, we carried out quantitative immunoblot analyses with antibodies directed against SinI or SinR, using as standards purified SinR and SinI proteins that had been tagged with histidine (His_6–SinI and His_6–SinR). The results show that the cellular concentration of SinI (~50 molecules/cell) was 18-fold lower than that of SinR (~900 molecules/cell) in cells reaching early stationary phase (Fig. 1B and C), the time at which derepression of SinR-controlled genes commences (Kearns

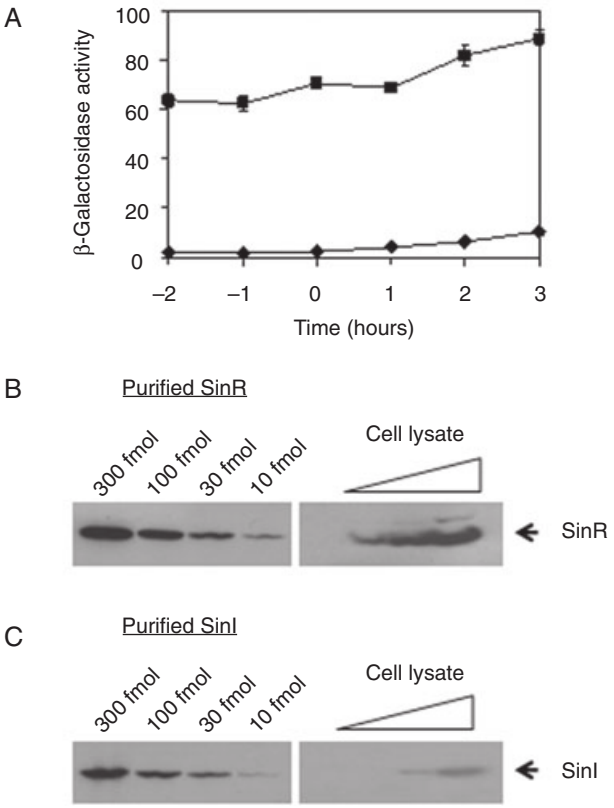

Fig. 1. SinI levels greatly exceed that of SinR.
A. Assays of β-galactosidase-specific activity of cells carrying either the P_{sinR}–*lacZ* (filled squares; strain YC108) or the P_{sinI}–*lacZ* (filled diamonds; strain YC127) fusion at the *amyE* locus on the chromosome. Assays were performed for cells grown in MSgg medium and harvested at the indicated times. Time zero refers to the end of exponential phase growth.
B and C. Quantitative immunoblots of SinR and SinI. Left-hand panels show affinity-purified, recombinant SinR and SinI proteins that were loaded at the indicated amounts. In the right-hand panels, cleared protein lysates prepared from early stationary phase cultures (1 h into stationary phase) were loaded on the same gel in a series of dilutions.

et al., 2005). Thus, the concentration of SinI would appear to be too low to counteract SinR effectively.

Cell population heterogeneity could explain the SinI/SinR paradox

A clue to resolving the paradox comes from the transcriptional regulation of *sinR* and *sinI*. Previous work has shown that *sinR* is expressed constitutively from a σ^A-dependent promoter whereas *sinI* is under the control of Spo0A, the master regulator for sporulation (Gaur *et al*., 1988; Shafikhani *et al*., 2002). Spo0A is known to be active in only a subset of cells in the population (Chung *et al*., 1994; Gonzalez-Pastor *et al*., 2003; Fujita and Losick, 2005). Thus, under conditions that activate Spo0A, the culture bifurcates into a subpopulation of Spo0A-ON cells

and a subpopulation of Spo0A-OFF cells. This cell population heterogeneity is believed to be governed by a bistable switch involving the phosphorelay and positive feedback loops that control the synthesis and phosphorylation of Spo0A (Burbulys *et al*., 1991; Stephenson and Hoch, 2002; Veening *et al*., 2005; V2006). [Actually, this is not a simple ON/OFF switch. Rather, when Spo0A is switched ON, the level of Spo0A increases in a graded manner over time with target promoters responding rapidly or slowly depending on their affinity for Spo0A (Jiang *et al*., 2000; Fujita and Losick, 2005). However, for present purposes we will simply consider Spo0A to be subject to an ON/OFF switch.] These considerations led us to hypothesize that SinR is produced more or less uniformly in all cells but that SinI is produced in only the subpopulation of Spo0A-ON cells. In other words, the protein measurements of Fig. 1 reflected the relative abundance of SinI and SinR averaged over the entire population. We also hypothesize that in those cells that are producing SinI, the protein antagonist reaches a sufficient concentration to overcome SinR-mediated repression. If so, then it further follows that only some cells in the population produce the polysaccharide and protein components of the extracellular matrix for the entire community. Indeed, earlier work has shown that mutants of the *eps* and *yqxM* operons can complement each other extracellularly, a finding that demonstrates that matrix components can be shared between cells (Branda *et al*., 2006).

sinI *is expressed in a subpopulation of cells*

To test our hypothesis, we fused the promoters for *sinI* and *sinR* to the gene (*gfp*) for the green fluorescent protein (GFP). The P_{sinI}–*gfp* and P_{sinR}–*gfp* fusions were then integrated into the chromosome at the *amyE* locus of strain 3610, a wild strain of *B. subtilis* that forms architecturally complex biofilms (Branda *et al*., 2001). Cells were grown to early stationary phase in MSgg medium and cell samples were prepared for fluorescent microscopy as described in the *Experimental procedures*. The results (Fig. 2A, left-hand panel) show that P_{sinR}–*gfp* was expressed in nearly all cells whereas P_{sinI}–*gfp* was expressed in only a small number of cells (the fraction of P_{sinI}–*gfp*-expressing cells being about 0.02; Fig. 2A, middle panel; Fig. 2C). It follows that if only about 2% of the cells are expressing *sinI*, then in those cells the level of SinI would exceed that of SinR; that is, the expected ratio of SinI to SinR would be roughly 2.8, which we calculate from the ratio of the concentration of SinI to SinR in the total population (1/18) divided by the fraction of cells that are producing SinI (0.02).

Confirming that *sinI* expression is indeed under Spo0A control, the results of Fig. 2A (right-hand panel) further show that little or no fluorescence from P_{sinI}–*gfp* was

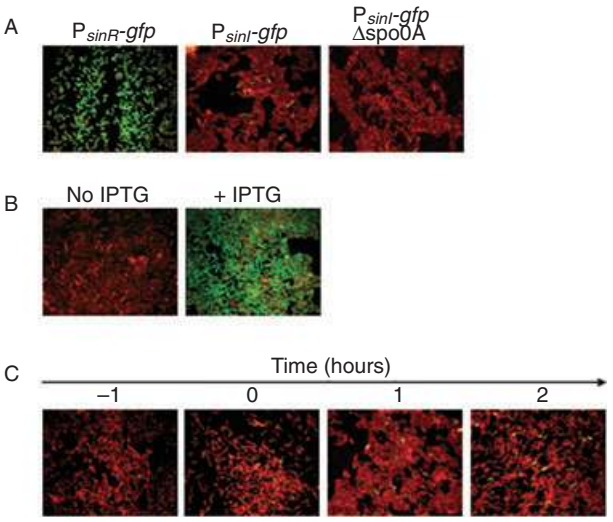

Fig. 2. Fluorescence microscopy of cells expressing P_{sinR}–*gfp* and P_{sinI}–*gfp*. In all panels, cells were treated with the red membrane stain FM4-64. Green is fluorescence from GFP.
A. Cells containing P_{sinR}–*gfp* (YC173) or P_{sinI}–*gfp* (YC162) integrated into the chromosome at the *amyE* locus of strain 3610 at 1 h after the end of exponential phase in MSgg medium.
B. Cells containing P_{sinI}–*gfp* and a construct (YC170) in which the *spo0A* gene was replaced with the mutant allele *sad67* under the control of an IPTG-inducible promoter.
C. Cells containing P_{sinI}–*gfp* (YC162) at the indicated times before and after the end of exponential phase growth in MSgg medium.

observed in cells of a *spo0A* mutant. Furthermore, the following experiment is consistent with the idea that cell population heterogeneity of Spo0A activation is the basis for the expression of *sinI* in only a subpopulation of cells. For this experiment, we replaced the wild-type *spo0A* gene with the mutant allele *sad67*, which encodes a truncated form of Spo0A whose activity does not depend on phosphorylation (Ireton *et al*., 1993). The *sad67* gene was under the control of an IPTG (isopropyl β-D-1-thiogalactopyranoside)-inducible promoter and was introduced into a strain that carried P_{sinI}–*gfp* at the *amyE* locus. Therefore, the use of this construct bypassed the normal control mechanisms that govern Spo0A synthesis and phosphorylation. The results of Fig. 2B (left-hand panel) show that when inducer was absent, little or no *sinI* expression was observed. However, shortly after IPTG was added to the medium (to a final concentration of 1 mM), *sinI* expression was observed in almost all cells (Fig. 2B, right-hand panel).

Expression of sinI *varies in different media*

Spo0A is known to be activated to high levels in difco sporulation (DS) medium (Jiang *et al*., 2000; Fujita and Losick, 2005). Yet, *B. subtilis* 3610 does not form thick pellicles in standing DS medium (data not shown) or architecturally complex colonies on solid DS medium (Fig. 3A,

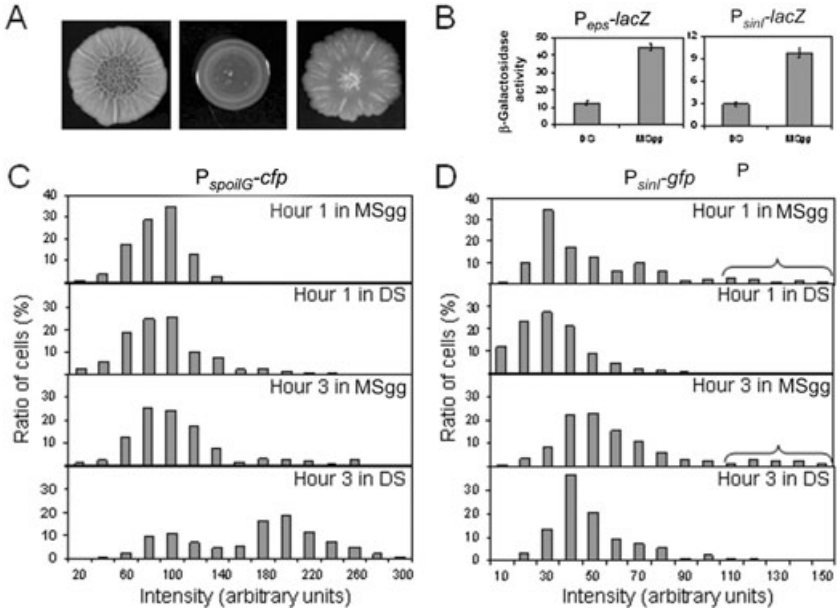

Fig. 3. Effect of medium on expression of *sinI*, *eps* and *spoIIG*.
A. Morphology of colonies of strain 3610 formed on agar plates containing MSgg (left-hand panel) or DS (middle panel) medium. The colony shown in the right-hand panel was formed on solid DS medium by a 3610 derivative overexpressing a functional fusion of SinI with GFP (YC227).
B. Assays of β-galactosidase-specific activity for P_{eps}–*lacZ* (left-hand panel; YC130) and P_{sinI}–*lacZ* (right-hand panel; YC127) in either DS or MSgg medium. Activities of P_{eps}–*lacZ* and P_{sinI}–*lacZ* were measured 1 h after the end of exponential phase growth.
C and D. Distribution of cells expressing P_{spoIIG}–*cfp* (columns in 3C; FC476) or P_{sinI}–*gfp* (columns in 3D; YC162) at various intensities in the indicated media and at the indicated times. Intensities for individual cells were measured using Metamorph. Each bar represents the percentage (*y*-axis) of cells whose fluorescence intensity was within ±5 in C and ±10 in D to the unit shown in the *x*-axis, versus total cells in the population. The horizontal bracket in D identifies a subpopulation of cells that expressed P_{sinI}–*gfp* highly.

left-hand and middle panels). How can this medium effect be explained? The results of Fig. 3B show that the medium effect is reflected in the expression levels of the extracellular matrix operon *eps* (left-hand panel) and the *sinI* gene (right-hand panel). Thus, transcription from the *eps* promoter as judged by using a P_{eps}–*lacZ* fusion was fourfold higher in MSgg medium than that in DS medium (Fig. 3B, left-hand panel). Likewise, transcription from the *sinI* promoter as measured using the P_{sinI}–*lacZ* fusion described above was higher in MSgg than in DS (Fig. 3B, right-hand panel). We infer that MSgg promotes biofilm formation more robustly than does DS because *sinI* is expressed at a higher level in the biofilm medium than in the sporulation medium. Reinforcing this view, colonies of cells engineered to overexpress SinI (as a functional fusion with GFP) grown on DS medium partially mimicked the complex colony architecture characteristic of MSgg-grown colonies (Fig. 3A, right-hand panel).

In light of these findings, we decided to compare the levels of Spo0A-directed gene expression in MSgg and DS media using a *cfp* fusion to the promoter for the sporulation operon *spoIIG* (P_{spoIIG}). P_{spoIIG} is known to have a relatively low affinity for the response regulator and is activated only when cells have attained a high level of Spo0A activity (Baldus *et al*., 1994; Fujita *et al*., 2005). As seen in Fig. 3C, a substantial subpopulation of cells could be seen by hour 3 of sporulation in DS medium that were actively expressing the P_{spoIIG}–*cfp* fusion. In contrast, few cells expressing P_{spoIIG}–*cfp* at a high level were seen in MSgg medium at either hour 1 or hour 3. Figure 3D shows that sharply different results were obtained with P_{sinI}–*gfp*; even at hour 3, few cells expressing P_{sinI}–*gfp* at high levels were seen in DS medium. However, in MSgg medium, a small proportion of cells expressed *sinI* at high levels at both hour 1 and hour 3.

eps *and* yqxM *are expressed in a subpopulation of cells*

Our finding that *sinI* is highly expressed only in a small population of cells (Fig. 2A) predicts that transcription of the *eps* and *yqxM* operons would similarly be limited to a subpopulation of cells. In confirmation of this expectation, fluorescent microscopy experiments using cells carrying a *gfp* or a *cfp* fusion to the promoters for *eps* (P_{eps}–*gfp*) and *yqxM* (P_{yqxM}–*cfp*) showed that both fusions were expressed in only a subset of cells (Fig. 4A and B). Furthermore, when a null mutation of *sinR* was introduced into the fusion-bearing strains, cell population heterogeneity was eliminated and almost all cells in the population were observed to express P_{eps}–*gfp* and P_{yqxM}–*cfp* (Fig. 4C

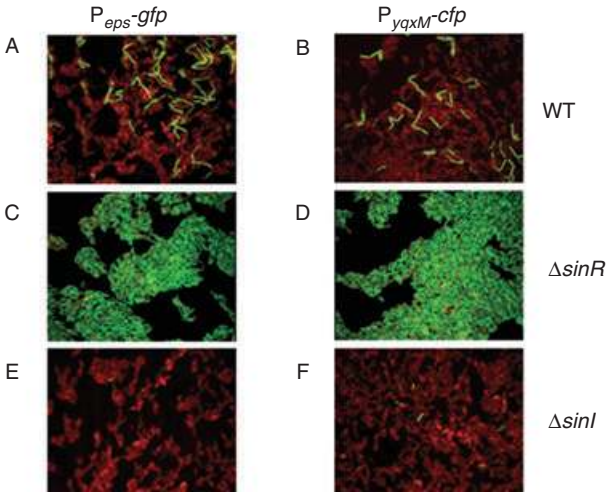

Fig. 4. Fluorescence microscopy of cells expressing P_{eps}–*gfp* or P_{yqxM}–*cfp*. The P_{eps}–*gfp* or P_{yqxM}–*cfp* was in a wild background (YC164 or YC189) or in a *sinR* (YC167 or YC221) or *sinI* (YC168 or YC190) mutant background. The cells were treated with the red membrane stain FM4-64. Fluorescence from P_{yqxM}–*cfp* was false-coloured green.

and D). In contrast, when a *sinI* mutation was introduced into the fusion-bearing strains, the proportion of cells expressing P_{eps}–*gfp* and P_{yqxM}–*cfp* was greatly reduced (Fig. 4E and F). We conclude that expression of the matrix operons is subject to cell population heterogeneity and that this heterogeneity is dependent on SinI-mediated relief from repression by SinR.

Finally, to extend the analysis further, we asked whether in the case of *yqxM*, the expressing cells corresponded to the same cells that were expressing *sinI*. To address this question, we carried out a dual-labelling experiment using a fusion of *cfp* (false-coloured green in Fig. 5) to the promoter for *sinI* and a *yfp* fusion (false-coloured red in Fig. 5) to the promoter for *yqxM.* The P_{sinI}–*cfp* fusion was introduced into the chromosome at the *thrC* locus and the P_{yqxM}–*yfp* fusion at the *amyE* locus.

The results showed that in cells highly expressing P_{sinI}–*cfp*, the expression of P_{yqxM}–*yfp* was also generally very strong (Fig. 5, left-hand column). Nevertheless, some cells could be observed that expressed P_{yqxM}–*yfp* but seemingly not P_{sinI}–*cfp.* Conversely, a few cells weakly expressing P_{sinI}–*cfp* were observed that were not measurably expressing P_{yqxM}–*yfp*. That some cells exhibited P_{yqxM}–*yfp* but not P_{sinI}–*cfp* expression could indicate that transcription of *sinI* is transient and that transcription from the matrix operon persists after the *sinI* gene is no longer expressed. Conversely, the cells that were weakly expressing P_{sinI}–*cfp* but not P_{yqxM}–*yfp* may simply represent cells that have not yet accumulated enough SinI to derepress the *yqxM* operon.

As a comparison, we also created a control strain harbouring the P_{yqxM}–*yfp* fusion and a *cfp* fusion to the promoter for *sinR* (P_{sinR}–*cfp*). The results of Fig. 5 (right-hand column) show that P_{sinR}–*cfp* was expressed in more or less all cells in the population whereas P_{yqxM}–*yfp* was expressed in only a subpopulation.

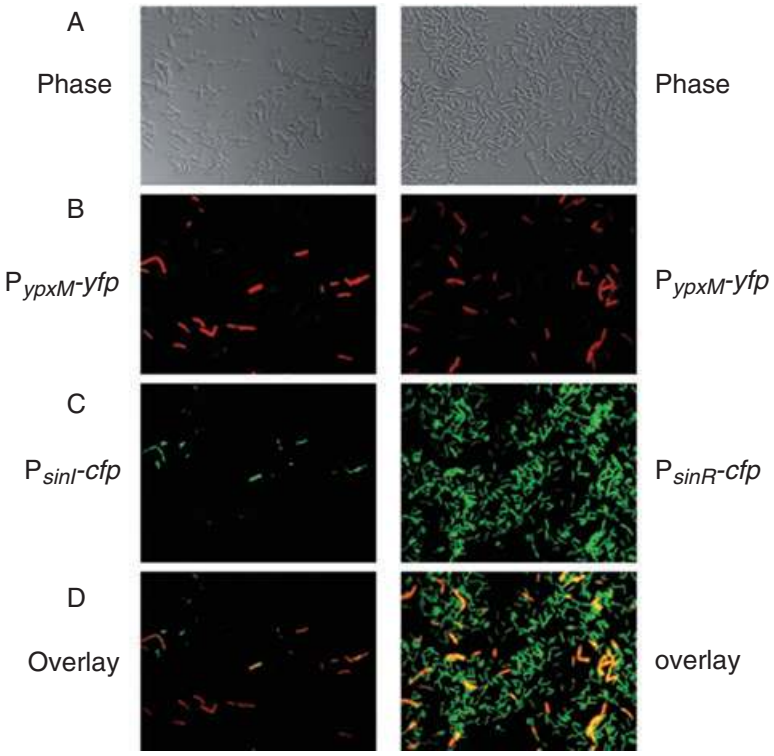

Fig. 5. Fluorescence microscopy of cells harbouring both P_{yqxM}–*yfp* and either P_{sinI}–*cfp* (YC243) or P_{sinR}–*cfp* (YC244). Fluorescence from P_{yqxM}–*yfp* was false-coloured red. Fluorescence from P_{sinI}–*cfp* and P_{sinR}–*cfp* was false-coloured green. Images in D are overlays from the corresponding images in B and C.

In toto the results of the dual-labelling experiments demonstrate that expression of the *yqxM* matrix operon does indeed exhibit cell population heterogeneity. Moreover, the results are consistent with the idea that this heterogeneity can be wholly, or at least partly, attributed to heterogeneity in the expression of *sinI*. Nevertheless, because we could not demonstrate a one-to-one correlation of *sinI* and *yqxM* expression in all cases, we do not rule out the possibility that additional as yet undefined levels of regulation contribute to cell population heterogeneity in matrix operon expression.

Discussion

Cell fate is generally thought of as being deterministic. That is, the fate that cells adopt is in most cases governed by the history of the cell or its proximity to inductive signals from other cells. However, an increasing number of cases are now known in which cell fate is controlled by stochastic processes. Examples of cell fate that are governed by stochastic mechanisms are entry into the persister state in *Escherichia coli* (Balaban *et al*., 2004, Lewis, 2007), and several cases in *B. subtilis* (Chung *et al*., 1994; Kearns and Losick, 2005; Dubnau and Losick, 2006; Maamar *et al*., 2007), including entry into the state of genetic competence, the choice between swimming and chains of sessile states, and entry into sporulation. The decision to enter sporulation is governed by a noise-driven, bistable switch controlling the accumulation of the transcription factor Spo0A~P (Veening *et al*., 2005; 2006). A further twist to the sporulation case is that the activation of Spo0A~P not only eventually leads to spore formation but first unleashes a cannibalistic like process in which cells that have activated Spo0A~P kill sibling cells that have not activated the transcription factor (Gonzalez-Pastor *et al*., 2003; Ellermeier *et al*., 2006). The nutrients thereby released stall sporulation. Therefore, Spo0A~P both promotes sporulation and indirectly impedes it. Cannibalism may be a mechanism to help ensure that cells do not commit to sporulation in response to what proves to be only a transient depletion of nutrients. Here we show that bistable control of Spo0A~P plays an additional role in the control of genes involved in production of extracellular matrix.

The principal finding of our investigation is that activation of *sinI*, which triggers derepression of the matrix operons *eps* and *yqxM*, occurs in only a subpopulation of the cells. In contrast, *sinR*, whose product is the target of SinI, is expressed more or less uniformly in the entire population. On this basis we propose that in the *sinI*-expressing cells, the concentration of the antirepressor reaches or exceeds that of the repressor, even though the overall level of SinI in the culture is much lower than that of SinR. It further follows from this that the matrix operons are derepressed only in a subpopulation of the cells, as confirmed by fluorescence microscopy using cells engineered to express fluorescent reporter genes joined to the promoters for the *eps* and *yqxM* operons. This implies that at the early stages of biofilm formation a subpopulation of cells are specialized for the production of matrix. Given earlier evidence indicating that matrix components can be exchanged between cells (Branda *et al*., 2006), it is tempting to speculate that the subpopulation of *sinI*-expressing cells is providing matrix components for the entire community.

The basis for this cell population heterogeneity is that *sinI* is under the positive control of Spo0A (Gaur *et al*., 1988; Shafikhani *et al*., 2002; Molle *et al*., 2003), whose synthesis and phosphorylation are known to be governed by a bistable switch (Veening *et al*., 2005). Consistent with this interpretation, cells engineered to produce a mutant form of Spo0A (Spo0A-Sad67) whose activity does not depend on phosphorylation (Ireton *et al*., 1993) expressed *sinI* in almost all cells in the population. Nonetheless, expression of *sinI* exhibited an unexpected medium dependence that seems at first glance to be at odds with the view that it is under the exclusive control of Spo0A~P. At 1 and 3 h after the end of exponential phase growth, a subpopulation of *sinI*-expressing cells was readily detected in the biofilm-promoting medium MSgg. In contrast, no *sinI*-expressing cells were detected at hour 1 and very few at hour 3 in the sporulation medium DS. Yet, growth in DS medium was much more effective in activating Spo0A than was growth in MSgg medium as judged from the expression of the promoter for the sporulation operon *spoIIG*. The *spoIIG* operon is under the direct positive control of Spo0A~P, but like other sporulation genes and operons under Spo0A~P control *spoIIG* has a relatively low affinity for the response regulator, representing a so-called high-threshold target of the response regulator (Fujita and Losick, 2005; Fujita *et al*., 2005). Hence, its expression requires relatively high levels of Spo0A~P. As seen in Fig. 3C, no *spoIIG*-expressing cells were observed at hour 1 in MSgg medium and relatively few at hour 1 in DS medium. However, by hour 3 in DS medium half or more of the cells were expressing *spoIIG*.

A possible clue to the basis for the dissimilar behaviours of *sinI* and *spoIIG* comes from an examination of the nucleotide sequence of the σ^A-controlled promoter governing *sinI* transcription (Y. Chai, unpubl. analysis). Just upstream of the –35 region is a perfect match to the consensus binding site for Spo0A~P (the so-called OA box), which presumably functions as a high-affinity, activation site for the response regulator (Shafikhani *et al*., 2002). However, just downstream of the start site are three imperfect matches to the OA box. We speculate that

these are operator sites at which Spo0A~P binding blocks the access of RNA polymerase to the promoter and thereby represses *sinI* but only when the response regulator reaches high enough concentrations to adhere to these postulated weak binding sites. We therefore posit that in MSgg medium Spo0A~P accumulates to intermediate levels that suffice to activate *sinI* but not repress it, thereby allowing for sustained synthesis of the antirepressor. In contrast, in DS medium Spo0A~P levels rapidly rise to levels that repress the biofilm-inducing gene. Our hypothesis therefore explains why MSgg medium but not DS medium promotes biofilm formation. Consistent with our hypothesis, colonies of cells engineered to overexpress *sinI* exhibited a rugose appearance on solid DS medium rather than the smooth-colony morphology characteristic of unengineered cells grown on the sporulation medium (Fig. 3A, middle and right-hand panels).

The goal of the current investigation was to study early events in the derepression of the extracellular matrix operons. For this purpose, we carried out our investigation using liquid cultures under conditions of continuous shaking in order to keep the cells dispersed and under uniform nutritional and aeration conditions. Normally, of course, cultures are not shaken during biofilm formation so that the cells can assemble into pellicles at the air/liquid interface or into colonies on solid medium. In light of our finding of the bifurcation of the culture into a subpopulation of *sinI*-expressing cells, it will be of high interest in future work to visualize the appearance, distribution and abundance of matrix-producing cells within the biofilm itself over the course of its formation.

Experimental procedures

Strains and media

Bacillus subtilis strains PY79, 3610 and other derivatives were grown in Luria–Bertani (LB), DS or MSgg (Branda *et al*., 2001), at 37°C or 22°C as indicated. *E. coli* strain DH5α was used as a host for molecular cloning and was grown at 37°C in LB medium. Approximately 1.5% agar was included when making solid agar medium. When appropriate, antibiotics were included at the following concentrations: 10 μg ml^{-1} of tetracycline, 100 μg ml^{-1} of spectinomycin, 20 μg ml^{-1} of kanamycin, 5 μg ml^{-1} of chloramphenicol and 1 μg ml^{-1} of erythromycin.

Colony morphology analysis

For colony architecture analysis on solid media, strains were grown to exponential growth phase in LB broth and 3 μl of cells was then applied to solid medium containing 1.5% Bacto agar. The plates were incubated at 22°C for 3 days. Images of the colonies on the plates were taken using a Nikon CoolPix 950 digital camera.

Strain construction

To construct strains with promoter–*lacZ* fusions integrated into the chromosome at the *amyE* locus, the promoter sequences of *sinI*, *sinR* and the *eps* operon were amplified by polymerase chain reaction (PCR). *B. subtilis* 3610 chromosomal DNA was used as the template in all PCR reactions, and oligonucleotides P_{sinI}-F1 and P_{sinI}-R1, P_{sinR}-F1 and P_{sinR}-R1, and P_{epsA}-F1 and P_{epsA}-R1 were used for amplifications of the promoter sequences of *sinI*, *sinR* and the *eps* operon respectively. PCR products were cloned into the plasmid pDG268 (Antoniewski *et al*., 1990). The resulting recombinant plasmids were then transformed into PY79 following the standard transformation protocol for *B. subtilis* (Gryczan *et al*., 1978). Transformants were selected for a double cross-over recombination at the *amyE* locus on the chromosome of PY79. The promoter–*lacZ* fusions at the *amyE* locus were then transferred from the PY79 background into the 3610 background using SPP1-mediated transduction as described previously (Yasbin and Young, 1974; Chu *et al*., 2006).

To construct promoter–*gfp* fusions, similar procedures as described above were applied except that oligonucleotides P_{sinI}-F2 and P_{sinI}-R2, P_{sinR}-F1 and P_{sinR}-R2, and P_{epsA}-F1 and P_{epsA}-R2 were used for amplification of the promoter sequences of *sinI*, *sinR* and the *eps* operon respectively. The amplified PCR products were cloned into another plasmid pYC121. Plasmid pYC121 contains a promoter-less *gfp* gene flanked by the *amyE* sequences and itself was constructed as follows: the *gfp* gene was amplified by PCR from the vector pNGFP (Chastanet and Losick, 2007) using oligonucleotides *gfp*-F1 and *gfp*-R1, and the PCR products were cloned into the HindIII and BamHI sites of the vector pDG1662 (Guérout-Fleury *et al*., 1996). The sequences of all PCR products were verified by DNA sequencing at Harvard DNA Sequencing Facilities.

For the construction of dual-labelled strains, the same promoter sequences of *sinI* and *sinR* to make promoter–*gfp* fusions were also cloned into plasmid pYC136 to generate promoter–*cfp* fusions. The recombinant plasmids were then transformed into PY79 and selected for a double cross-over recombination at the chromosomal *thrC* locus. The promoter–*cfp* fusions at the *thr*C locus were then transferred from the PY79 background into strain YC222, which contains a P_{yqxM}–*yfp* fusion integrated at the chromosomal *amyE* locus in 3610 (The P_{yqxM}–*yfp* fusion was a gift of H. Vlamakis and C. Aguilar).

All insertion deletion mutations were generated by long-flanking homology PCR that has been described previously (Wach, 1996). A detailed description of primers used in this work and all resulting strains is provided in Table S1.

β-Galactosidase assays

Cells were incubated in MSgg or DS medium at 37°C in a water bath with shaking. One millilitre of culture was collected at each time point. Cells were spun down and pellets were resuspended in 1 ml Z buffer (40 mM NaH_2PO_4, 60 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM KCl and 38 mM β-mercaptoethanol) supplemented with 200 μg ml^{-1} freshly made lysozyme. Resuspensions were incubated at 30°C for 15 min. Reactions were started by adding 200 μl of 4 mg ml^{-1}

ONPG (2-nitrophenyl β-D-galactopyranoside) and stopped by adding 500 µl of 1 M Na_2CO_3. Samples were briefly spun down. The soluble fractions were transferred to cuvettes (VWR), and OD_{420} values of the samples were recorded using a Pharmacia ultraspectrometer 2000. The β-galactosidase-specific activity was calculated according to the equation $(OD_{420}/\text{time} \times OD_{600}) \times \text{dilution factor} \times 1000$. Assays were conducted at least in duplicate.

Purification of His_6–SinR and His_6–SinI proteins

Escherichia coli strains RL4219 and RL4220 (Kearns *et al.*, 2005) were used for the production of His_6–SinR and His_6–SinI fusion proteins respectively. Five hundred millilitres of cultures was grown in LB broth supplemented with 25 µg ml^{-1} kanamycin and 50 µg ml^{-1} chloramphenicol at 30°C to an OD_{600} of 0.5. IPTG was then added to a final concentration of 1 mM and cultures were incubated at 30°C for two more hours. Cells were harvested and washed once with 50 ml cold phosphate buffer (20 mM sodium phosphate, 200 mM NaCl, 10% glycerol, 1 mM PMSF, pH 7.4). Cell pellets were suspended in 5 ml of cold phosphate buffer supplemented with 200 µg ml^{-1} of freshly made lysozyme solution and incubated on ice for 30 min. Lysed cells were further disrupted on ice using sonication. Cell lysates were centrifuged at 5000 r.p.m. for 5 min to remove cell debris and were further ultracentrifuged at 35 000 r.p.m. for 30 min at 4°C. Soluble fractions were transferred to clean cold tubes.

One millilitre of Ni-NTA agarose beads (Qiagen) was added to the cleared lysate and samples were gently rotated for 2 h at 4°C. The lysate/bead mixture was then loaded onto a column and washed five times, each time with two bed volumes of wash buffer (20 mM sodium phosphate, 300 mM NaCl, 10% glycerol, 20 mM imidazole, pH 8.5). The column was eluted with five bed volumes of elution buffer (20 mM sodium phosphate, 300 mM NaCl, 10% glycerol, 300 mM imidazole, pH 8.5). Collected samples were dialysed against 20 mM sodium phosphate, 300 mM NaCl, 0.3 mM DTT, 10% glycerol, pH 7.4 and were quantified using a BCA Protein Assay Kit by Pierce (IL, USA). Proteins were aliquoted and stored in 50% glycerol at –80°C.

Quantitative immunoblots

Protein lysates were diluted, mixed 2:1 with protein lysis buffer (Bio-Rad) and were size-fractionated by 15% SDS-PAGE. In parallel, affinity-purified His_6–SinR and His_6–SinI proteins were diluted in the same way and loaded on the same SDS-PAGE. After size-fractionation, proteins were transferred to an immunoblot-specific PVMF membrane (Bio-Rad) at 250 mA for 2 h using a Bio-Rad mini-PROTEIN II Cell. After completion of protein transfer, the membrane was briefly washed with TBS buffer (20 mM Tris-Cl, 200 mM NaCl, 0.1% Tween 20, pH 7.5) and incubated in 30 ml TBS buffer supplemented with 5% skim milk. The membrane was then transferred to a clean tip box and incubated with the affinity-purified antiserum against either SinI or SinR at a dilution of 1:10 000 in 15 ml TBS buffer. After 3 h incubation at room temperature with gentle shaking, the membrane was then washed three times, each time with 30 ml TBS buffer. A second goat anti-rabbit antibody (Bio-Rad) was added to the membrane with a dilution of 1:6000 in 15 ml TBS buffer and incubated at room temperature for 1 h. The membrane was again washed three times with TBS buffer. Finally, the membrane was transferred to a clean tip box, mixed with 7 ml each of Solutions A and B in the SuperSignal West Pico Chemiluminescent Kit (Pierce), and incubated for 5 min at room temperature. An X-ray film was exposed to the membrane and then developed using an Eastman Kodak RP X-OMAT processor.

To quantify the number of SinI molecules per cell, we estimated that the pixel density from the lane containing 10 fmol of purified His_6–SinI was roughly equal to that from the middle lane on the right panel (Fig. 2C), which contained protein prepared from a total of 1.2×10^{11} cells. It was thus calculated that for each cell, there are about 50 molecules of SinI. The number of SinR molecules per cell was quantified in a similar way. We estimated that the pixel density from the middle lane on the right panel (Fig. 2B), which contained protein prepared from a total of 6×10^{10} cells, would have been equal to 90 fmol of purified His_6–SinR. We thus calculated that there are about 900 SinR molecules per cell.

Fluorescence microscopy

Cells were grown in MSgg broth to early stationary phase. One millilitre of the culture was harvested and centrifuged. Cells were washed with PBS buffer twice (PBS buffer was autoclaved and filtered through a 0.25 µM syringe filter prior to use), and resuspended in 50 µl PBS buffer. Three microlitres of resuspended cells was dropped on the center of an agar-coated microscopy slide (VWR, catalogue number 48311-702), and covered by a 0.15 mm microscopy cover slide (VWR, catalogue number 48366-045). Cover slides were pretreated with polyl-lysine. Samples were examined using an Olympus workstation BX61. Images were taken and analysed using an automated software program MetaMorph (Universal Imaging Corporation).

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Supplementary material

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