SinI modulates the activity of SinR, a developmental switch protein of *Bacillus subtilis*, by protein–protein interaction

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SinR, a 111-amino-acid DNA-binding protein, is a pleiotropic regulator of several late growth processes in *Bacillus subtilis*. It acts as a developmental switch, positively regulating genes for competence and motility and repressing *aprE* and stage II sporulation genes. It is encoded by the second gene in a two gene operon, but previous results have also indicated that these two genes are differently regulated. We show in this discussion that the product of *sinI*, the first open reading frame (ORF) of this operon, interferes with the function of SinR. In vivo experiments have demonstrated that overexpression of *sinI* results in phenotypes that are observed in cells with a null mutation of *sinR*. A chromosomal in-frame deletion of *sinI* gives rise to a phenotype associated with higher levels of SinR. Thus, SinI acts as an antagonist to SinR. In vitro experiments have shown that the interaction between these two proteins is a direct one. SinI prevents SinR from binding to its target sequence on *aprE*, and the two proteins form a complex that can be immunoprecipitated with antibodies to either SinR or SinI.

[Key Words: Sporulation; post-translational control; protein–protein interaction]

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Nutrient depletion initiates a series of responses in the soil bacterium *Bacillus subtilis* that enables it to survive under adverse conditions. Among these responses are the synthesis and secretion of degradative enzymes and antibiotics, the development of motility and competence and, finally, the appearance of spores (Smith 1992). These processes have overlapping control mechanisms, but they can be differentiated on the basis of specific genetic and nutrient requirements; for example, glucose is essential for competence but represses sporulation. Several proteins affecting these pathways have been described (Smith 1993). SinR (formerly known as Sin), a DNA-binding protein, is one of these regulators of developmental processes that are initiated at the end of exponential growth, in response to nutrient depletion (Smith 1989). Previous work has shown that SinR is a repressor of *aprE*, the gene coding for the secreted protease, subtilisin, and it also inhibits sporulation (Gaur et al. 1986, 1991). It has recently been shown that SinR exerts its repressive effects on sporulation immediately before stage II, by inhibiting the expression of *spoIIA*, *spoIIE*, and *spoIIG* (Mandic-Mulec et al. 1992). On the other hand, null mutations in the *sinR* gene prevent development of competence and motility and the production of growth-related autolysins (Gaur et al. 1986; Sekiguchi et al. 1988). It has been shown that its requirement for the development of motility is at the level of motility regu-

lon gene expression (M. Predich, M. Zelic, and I. Smith, unpubl.). Thus, SinR acts as a developmental switch, controlling alternate developmental pathways. Given this role, the concentrations or function of this protein must be tightly regulated.

We have shown previously that levels of SinR, as measured by immunoassay, actually increase at a time when its stage II negative target genes showed increased transcription (Mandic-Mulec et al. 1992). These results suggested that SinR function may be regulated at a post-translational level. The *sin* operon, controlled by two upstream promoters, P1 and P2, consists of two adjacent open reading frames (ORFs), the downstream *sinR* and ORF-1, now called *sinI*. Recent experiments have indicated that promoter P3, between *sinI* and *sinR* can also function in vivo. Studies with translational *lacZ* fusions of *sinR* and *sinI* have shown that these genes are regulated differently (Gaur et al. 1988). *sinI* is under developmental control, being expressed at low levels during vegetative growth and at high levels during sporulation. Its expression requires the essential early sporulation genes *spoOA* and *spoOH*, and it is repressed by glucose, which blocks the early stages of sporulation. On the other hand, *sinR* is expressed at a similar low level throughout growth and its expression increases only slightly at the end of exponential growth (Gaur et al. 1988; Mandic-Mulec et al. 1992). *sinR* gene activity is

not affected by any of the above genetic or nutrient factors that control *sinI* expression. In addition, greater sporulation inhibition was observed when plasmids contained only the *sinR* gene rather than the entire *sin* operon. Finally, multicopy plasmids containing *sinI* (but with mutations in *sinR*) caused the appearance of colonies that were rougher than wild type and resembled those in which the chromosomal *sinR* gene had been disrupted. These results indicated that *sinI* may be acting as an antagonist of SinR.

In vivo data presented in this discussion support this observation. In addition, we have overexpressed and purified SinI. We have demonstrated by functional SinR assays and immunoprecipitation experiments that SinI and SinR interact physically with each other and that a direct protein–protein association is involved in the control of SinR activity.

Results

As discussed, several lines of evidence indicated that SinR function during development may be regulated by SinI. To provide additional evidence for this hypothesis, two types of experiments were performed. We first overexpressed the *sinI* gene in vivo and measured several cellular processes that have been shown to be regulated by SinR. Second, we inactivated the chromosomal *sinI* gene and investigated the results of this mutation on the same SinR-dependent processes.

Overexpression of *sinI* is similar to disruption of *sinR* in the chromosome

To test whether *sinI* was specifically responsible for the rough phenotype observed in cells carrying the *sin* operon (both *sinI* and *sinR*) on multicopy plasmids, but with the *sinR* inactivated, we cloned *sinI* by itself on a multicopy plasmid, resulting in pIS256. IS1576, a strain carrying pIS256, sporulated well, but colonies were extremely rough. We examined the microscopic appearance of this strain after overnight growth in nutrient sporulation medium (NSM). The cells displayed an unusual aggregation in the form of long filamentous septate chains (Fig. 1C) when compared with wild-type cells, IS75 (Fig. 1A). Placing the *sinI* gene behind the inducible pSPAC promoter and adding IPTG to a strain containing this construct integrated into the chromosome (IS791) gave a similar phenotype (Fig. 1D). These septate chains were similar to those observed when *sinR* was inactivated in the chromosome, for example, IS720 (Fig. 1B). Unlike the wild-type strain, the cells shown in Figure 1, B–D, were nonmotile, and on plates, colonies were very rough.

We then measured the levels of competence in strains overexpressing *sinI*, as *sinR* is required for competence (Gaur et al. 1986; Albano et al. 1987). There was a drastic reduction of competence in IS1576, carrying pIS256, of approximately three orders of magnitude, as measured by the number of transformants. Similar results were obtained with strain IS791, carrying an integrated *sinI* gene behind the pSPAC promoter, after IPTG induction

Figure 1. Photomicrograph of otherwise isogenic strains of *B. subtilis* in NSM. The cells were grown in NSM overnight at 32°C and were examined and photographed at 800× magnification, using phase-contrast optics (40× objective). (A) IS75 (wild type); (B) IS720 (chromosomal *sinR* null mutation); (C) IS1576 (*sinI* on a multicopy plasmid); (D) IS791 (*sinI* behind an inducible pSPAC promoter integrated into the chromosome and induced with IPTG).

(data not shown). Because cells overexpressing *sinI* tend to aggregate, giving inaccurate viable counts, a more accurate measurement of competence was made by studying the effects of *sinI* overexpression on the late competence gene *comG* (Albano et al. 1987). pIS256 was introduced into a strain containing a *comG*–*lacZ* fusion (integrated at the *amyE* locus), and β-galactosidase-specific activity was measured by growth in liquid minimal salts competence medium. The results are shown in Figure 2. *comG* expression was reduced approximately threefold in IS1593, carrying pIS256, as compared with IS1594, which carries pIS257, a plasmid with a deletion in *sinI*.

We then investigated the effects of SinI overproduction on *spoIIA*, a stage II sporulation gene that is repressed by SinR (Mandic-Mulec et al. 1992). For this study we used IS820, a strain with a *spoIIA*–*lacZ* fusion and also carrying the *sinI* gene behind the pSPAC promoter, integrated into the chromosome. The expression of *spoIIA* was measured in IS820 after inducing the culture with IPTG. As shown in Figure 3, *spoIIA* was overexpressed at a level fourfold higher than wild-type cells (IS819). Similar quantitative results have been obtained with *spoIIA*–*lacZ* fusions when the chromosomal *sinR* gene was inactivated (Mandic-Mulec et al. 1992).

The above results, summarized in Table 1, indicate that overexpression of *sinI* caused a pleiotropic phenotype resembling that observed when the chromosomal *sinR* gene was inactivated. The fact that these effects were observed both with multicopy *sinI* and with the *sinI* gene integrated into the chromosome behind an inducible promoter indicated that the *sinI* gene product was responsible for the observed phenotypes.

Figure 2. Effect of *sinI* on *comG-lacZ* expression. β -Gal was assayed in strains containing an integrated *comG-lacZ* fusion (IS803) grown in minimal salts competence medium, and samples were withdrawn at indicated times. (●) IS1593 (carrying pIS256 with an intact *sinI*); (○) IS1594 (carrying pIS257 with a deletion in *sinI*). (T_0) The time at which the cells entered the stationary phase of growth; (T_{-1}) an hour before T_0 ; (T_{+1}) an hour after T_0 , etc.

Inactivation of *sinI* results in a *SinR*⁺ phenotype

Because overexpression of *sinI* resulted in a phenotype similar to that observed in chromosomal *sinR* null mutants, we wanted to examine the effect of a *sinI* chromosomal deletion. According to our hypothesis about the role of SinI, it was predicted that cells with a disrupted *sinI* gene would behave as though SinR were overpro-

Figure 3. Effect of *sinI* on *spoIIA-lacZ* expression. Expression of *spoIIA* was assayed by measuring β -gal activity of *spoIIA-lacZ* fusions, in otherwise isogenic strains, during growth in NSM. (□) IS820 (*sinI* behind a pSPAC promoter and induced with IPTG); (○) IS819 (wild type); (●) IS879 (with an *sinI* chromosomal null mutation). IPTG was added when the culture was inoculated.

Table 1. Comparison of phenotypic effects of *sinI* overexpression and *sinR* deletions

	Overexpression of <i>sinI</i>	<i>sinR</i> deletion in the chromosome
Colonial morphology	rough	rough
Sporulation	<i>spoIIA</i> : fourfold overexpressed	<i>spoIIA</i> : fourfold overexpressed
Competence	3 log orders decreased <i>comG</i> expression reduced	3 log orders decreased <i>comG</i> expression reduced
Motility	nonmotile	nonmotile
Microscopic examination	long filamentous chains with septations	long filamentous chains with septations

duced (a *SinR*⁺ phenotype). A nonpolar, in-frame deletion was made by deleting 33 codons from the center of the *sinI* ORF in pIS256, creating pIS257. This plasmid-borne *sinI* deletion in pIS257 was then integrated into the chromosome by gene conversion, as described in Materials and methods. The resulting strain, IS874, had a smooth translucent colonial morphology, and its sporulation was reduced to 1–10% of wild-type levels. Similar sporulation results were obtained when the chromosomal *sinI* was disrupted with a kanamycin resistance cassette (data not shown). Because, as shown above, *sinI* activated the expression of *spoIIA* fourfold, we asked whether a *sinI* deletion would cause lowered *spoIIA* expression. The results are shown in Figure 3. *spoIIA-lacZ* activity was repressed in IS879, carrying the in-frame deletion of *sinI* in the chromosome, compared with a wild-type strain (IS819).

We have shown previously that overproduction of SinR inhibits the production of extracellular proteases (Gaur et al. 1986). We assayed the production of extracellular protease activity in a strain carrying an inactivated *sinI* gene, IS880. In the same experiment, for comparison, we also measured extracellular protease activity in a strain carrying the *sinR* gene on a multicopy plasmid (IS1491). Very little exoprotease activity was observed in these strains compared with the amounts observed in a control strain (IS812) (Fig. 4).

Thus, the cells with disrupted *sinI* behaved as though SinR were overproduced. The results are summarized in Table 2. This *SinR*⁺ phenotype observed in strains with a deletion of *sinI* could be eliminated by reintegrating a wild-type *sinI* at the region of homology, providing SinI in *trans* on a multicopy plasmid, or by inducing the strain carrying the pSPAC *sinI* construct. This indicated that the phenotype was caused by the disruption in *sinI*.

SinR levels are not significantly affected by *sinI* expression

The above results clearly indicate that SinI controlled the activity of *sinR*, but the nature of this interaction was unknown. It was possible that SinI modulated *sinR*

Figure 4. Effect of *sinI* disruption on extracellular protease production. Cells were grown in NSM, and extracellular protease activity was measured in cell-free supernatants of IS657 (a wild-type strain) (■), in IS1491 (carrying *sinR* on a multicopy plasmid) (□), and in IS880, having a deletion of *sinI* in the chromosome (▲). Activity is expressed in units, as described in Materials and methods.

gene expression or the stability of the SinR protein, which could explain the physiological data described above. To see whether overexpression of *sinI* or its absence alters cellular SinR levels, we measured SinR concentrations during growth in strains overexpressing *sinI* or in strains with a *sinI* deletion.

Cultures of IS1576, harboring a multicopy plasmid with *sinI*, and IS1556, a wild-type strain carrying a plasmid vector, were grown in NSM, and samples were withdrawn at regular intervals for immunodetection of SinR using polyclonal antibodies raised against the SinR protein. The results are shown in Figure 5A. The levels of SinR increased slightly during growth in both of these strains. Overexpression of *sinI* did not cause a reduction in the levels of SinR. The levels of SinR were actually higher in the strain overexpressing *sinI*. This effect presumably reflects the autogenous control of the *sin* operon by SinR (Smith et al. 1991; N. Gaur and I. Mandic-Mulec, unpubl.). A similar experiment was performed

Figure 5. Effect of *sinI* on SinR protein levels during growth. The strains were grown in NSM, and cell-free extracts were prepared and subjected to Western blot analysis as described in Materials and methods. (A) (Lanes A–E) SinR levels in IS1556, carrying vector plasmid pIS253, at times $T_{-0.25}$, T_0 , T_1 , T_2 , and T_3 , respectively. (Lane F) IS432, carrying a chromosomal deletion of *sinR*, at T_2 . (Lanes G–K) SinR levels in IS1576, carrying *sinI* on a multicopy plasmid pIS256, at times $T_{-0.25}$, T_0 , T_1 , T_2 , and T_3 , respectively. (Lane L) Purified SinR used as a positive control. (Lane M) Molecular mass standards. (B) (Lanes A–E) SinR levels in IS1595, a control strain, at $T_{-0.25}$, T_0 , T_1 , T_2 , and T_3 , respectively. (Lane F) IS432 at T_2 . (Lanes G–K) SinR levels in IS874, carrying an inactivated *sinI* in the chromosome, at $T_{-0.25}$, T_0 , T_1 , T_2 , and T_3 , respectively. (Lane L) Molecular mass standards. (Lane M) Purified SinR protein.

with IS874, a strain carrying a deletion of *sinI* in the chromosome, and a control strain, IS1595; the results are shown in Figure 5B. A deletion of *sinI* did not result in higher levels of SinR in vivo. Thus, the effects of *sinI* overexpression or absence could not be explained by the alteration of SinR levels.

SinI inhibits DNA binding of SinR to *aprE*

The finding that SinR levels are not affected significantly by overexpression or absence of *sinI* clearly indicated that *sinI* is able to regulate the activity of SinR at a post-translational step. To investigate the nature of this mechanism, we wanted to study interactions between SinR and SinI in vitro. We first overproduced SinI in *Escherichia coli*, using a bacteriophage T7 RNA polymerase-controlled system (Studier et al. 1990). We were able to purify SinI in one step, taking advantage of its low binding to a DEAE–Sephacel column at pH 8.2 (Fig. 6). We observed a protein of ~6 kD, which is consistent with the predicted molecular mass from the derived amino acid sequence. Interestingly, SinI also did not bind to DNA affinity columns used to purify DNA-binding proteins (Fig. 7; data not shown). The purified protein reacted with the antibody raised against a synthetic polypeptide containing the middle 33 amino acids of SinI. Furthermore, the amino acid sequence of the first 20 amino acids of the purified protein, as determined by

Table 2. Comparison of phenotypic effects of *sinI* deletions and *sinR* overexpression

	<i>sinI</i> deletion in the chromosome	Overexpression of <i>sinR</i>
Colonial morphology	smooth	smooth
Phenotype	Spo [−] (1–10% of wild type) <i>spoIIA</i> expression inhibited	Spo [−] (2–3 orders of magnitude decrease) <i>spoIIA</i> expression inhibited
Competence	competent	competent
Motility	normal	normal
Extracellular protease	10-fold decrease	10-fold decrease

Figure 6. (A) Purification of SinI. Samples from the purification steps were subjected to electrophoresis on a 12% SDS-polyacrylamide gel, and the gel was stained with Coomassie blue. (Lane 1) Fifty micrograms of crude extract of *E. coli* BL21(DE3)pLysS strain, harboring the vector pT7-1, after IPTG addition. (Lane 2) Fifty micrograms of crude extract of the above strain, but carrying the *sinI*-containing plasmid, pS281, after IPTG addition. (Lane 3) Three micrograms of SinI from pooled DEAE-Sephacel fractions, containing SinI. (Lane 4) Protein standards. The numbers at left indicate the migration (in kD) of these standards. (B) Immunochemical identification of SinI. Proteins from the extracts, as described above, were separated on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose filter that was analyzed by immunoblotting with antiserum against SinI. The molecular mass of the protein is seen at right. (Lane 1) Fifty micrograms of crude extract of *E. coli* BL21(DE3)pLysS, containing pT7-1. (Lane 2) Fifty micrograms of crude extract of BL21(DE3)pLysS containing pS281, after IPTG induction. (Lanes 3 and 4) Fractions from DEAE-Sephacel column of extract illustrated in lane 2.

micro-Edman degradation, was identical to that predicted from the SinI ORF (data not shown). To study the nature of the reaction between SinR and SinI, we then tested the effect of SinI on the binding of purified SinR (Gaur et al. 1991) to its target sequence on *aprE*, in a gel mobility-shift assay.

SinR binds to *aprE* in a region 220–260 bp upstream from the transcription start site (Gaur et al. 1991). In the studies reported here we used as a probe a 250-bp fragment of the *aprE* promoter and upstream regulatory sequences. Different amounts of SinI were incubated at room temperature for 15 min with constant levels of SinR. The mixtures were then used for the gel retardation studies, which were performed as described previously (Gaur et al. 1991). SinI prevented SinR from binding to *aprE* in a concentration-dependent manner (Fig. 7, lanes 4–9). Complete inhibition of SinR binding was observed in the presence of 0.5 μ M SinI, which corresponds to a molar ratio of 1 : 2 monomers of SinR/SinI. However, it is impossible to discuss the true stoichiometry of the interaction because SinR behaves in solution as a tetramer, and the actual state of multimerization of SinI

as purified is currently unknown. In addition, we have no estimate of the fraction of active molecules in each preparation. If SinR were allowed to bind to *aprE* (15 min at room temperature) and were then challenged with 0.5 μ M SinI at room temperature for 15 min, the retarded band could still be seen (lane 10). Thus, SinI is much less able to inhibit the binding of SinR to *aprE* once the SinR–DNA complex is formed. SinI, by itself, does not bind to the *aprE* probe (lane 2). These results suggest that SinI can associate with SinR by protein–protein interaction and thus attenuates its ability to bind to DNA. Control gel retardation experiments with the ArgR protein and DNA containing “arg boxes” showed that SinI had no effect on the interaction of this regulatory protein and its DNA-binding sites (data not shown).

SinI binds to SinR

The results of the *aprE*-binding studies presented in the previous section suggested that SinI regulates SinR function by direct protein–protein interaction. To see whether the two proteins formed a complex, we performed immunoprecipitation experiments. [35 S]Methionine-labeled SinI was synthesized in vitro using a bacterial cell-free-coupled transcription translation system. We observed a protein of 6 kD in the extract programmed with the SinI-containing plasmid pS281 (Fig. 8, lane 2). This protein was immunoprecipitated with SinI-specific antibodies (Fig. 8, lane 7). The radiolabeled SinI was added to SinR, and the complex was mixed with the same SinR antibody used for the experiments described in Figure 5. The resulting complexes were purified by binding to protein A–Sepharose beads and were then analyzed on SDS-polyacrylamide gels. The results of these experiments indicated that the SinR antibody could precipitate SinI in the presence of SinR (Fig. 8, lane 8) but not if SinR was absent (Fig. 8, lane 6). The addition of an

Figure 7. Effect of SinI on SinR binding to *aprE*. The 3' end-labeled 250-bp *DdeI*–*NotI* fragment of *aprE* containing the SinR-binding region was used as a probe. In all lanes where SinR was present, its concentration was 250 nM. (Lane 1) Free probe; (lane 2) probe + SinI (500 nM); (lane 3) probe + SinR; (lane 4) probe + SinR/SinI (15.63 nM); (lane 5) probe + SinR/SinI (31.25 nM); (lane 6) probe + SinR/SinI (62.5 nM); (lane 7) probe + SinR/SinI (125 nM); (lane 8) probe + SinR/SinI (250 nM); (lane 9) probe + SinR/SinI (500 nM); (lane 10) probe + SinR for 15 min at room temperature. SinI was then added to a final concentration of 500 nM. The retarded band is indicated by the open arrowhead.

Figure 8. Coimmunoprecipitation of SinI and SinR. ^{35}S -Labeled SinI was prepared using an S-30 extract of *E. coli*, as described in Materials and methods. Various samples, as described below, were fractionated on a 15% SDS–polyacrylamide gel followed by autoradiography. (Lane 1) In vitro-translated proteins of the vector pT7-1; (lane 2) ^{35}S -labeled SinI that was translated in the S-30 lysate carrying pIS281; (lane 3) in vitro-translated proteins of extracts containing pT7-1, subjected to immunoprecipitation with SinI antibodies; (lane 4) identical to lane 3, but using SinR antibodies; (lane 5) identical to lane 3, but SinR was added along with SinR antibodies; (lane 6) [^{35}S]methionine-labeled extracts containing pIS281, subjected to immunoprecipitation with SinR antibodies; (lane 7) identical to lane 6, but treated with SinI antibodies; (lane 8) identical to lane 6 but treated with SinR and SinR antibodies; (lane 9) identical to lane 8 but 1 μg of nonradioactive SinI was added before adding SinR and SinR antibodies. The open arrowhead indicates the position of a standard molecular mass protein that migrated to a position corresponding to ~ 6 kD.

excess of nonradioactive SinI to an immunoprecipitation reaction with SinR and SinR antibodies decreased the amount of radioactivity observed in the SinI band (cf. lanes 8 and 9), indicating that the labeled protein is SinI. The complementary experiment, using radioactive SinR, could not be performed because of difficulties in radiolabeling this protein in vitro. However, we were able to immunoprecipitate purified SinR with SinI antibodies in the presence of SinI as detected by Western immunoblotting (Fig. 9). We conclude that the SinR and SinI proteins bind directly to each other.

Discussion

SinR is a dual function regulatory protein exerting positive and negative effects on gene expression during *B. subtilis* late growth. It is needed for development of competence and motility and for the production of autolysin (Gaur et al. 1986; Sekiguchi et al. 1988). An increased expression of the *sinR* gene is inhibitory for sporulation and the production of extracellular proteases (Gaur et al. 1991; Mandic-Mulec et al. 1992). Thus, the function of

the protein has to be turned off for cells to sporulate. How is this achieved? It seems likely that in addition to complex transcriptional regulation (Gaur et al. 1988), the activity of SinR is modulated by SinI.

The in vivo results presented here indicate that *sinI* down-regulates SinR activity. Thus, when *sinI* was over-expressed, whether by being present on a multicopy plasmid or by means of an inducible promoter upstream from *sinI*, SinR functions, such as the development of competence and motility as well as the repression of stage II-specific sporulation genes, were inhibited. Similarly, the phenotypes associated with elevated levels of SinR, such as the inhibition of stage II-specific gene expression and lowered production of extracellular proteases, are observed when *sinI* is inactivated in the chromosome. Thus, we have demonstrated that these two factors are functionally antagonistic. In addition, we have shown biochemically that SinI and SinR interact directly with each other. This is corroborated by the fact that SinI prevents SinR from binding to its target site on the *aprE* promoter (Fig. 7) and also that SinR antibody can immunoprecipitate SinI when the latter protein is mixed with SinR (Fig. 8). Conversely, SinR is immunoprecipitated by antibodies to SinI when mixed with SinI (Fig. 9).

What role does SinI play in normally differentiating *B. subtilis*? *sinI* is under sporulation control, its expression increasing with the onset of sporulation, and this rise in expression is inhibited by mutations in *spoOH* and *spoOA*. The former codes for a σ -factor, σ^H (Dubnau et

Figure 9. Coimmunoprecipitation of SinI and SinR. Purified SinR was treated in various ways, described below, and then immunoprecipitated (lanes 2–5) with SinI or SinR antibodies. The precipitates were fractionated by 15% SDS-PAGE, and the proteins were transferred to nitrocellulose papers that were probed with SinR-specific antibodies. (Lane 1) One microgram of purified SinR was added directly to the gel before electrophoresis; (lane 2) purified SinR immunoprecipitated with SinR-specific antisera; (lane 3) SinR immunoprecipitated with SinI-specific antisera; (lane 4) SinR mixed with purified SinI and immunoprecipitated with SinI-specific antisera; (lane 5) SinI immunoprecipitated with SinI antibodies. The open arrowhead indicates the position of a standard molecular mass protein corresponding to ~ 14 kD.

al. 1988; Zuber et al. 1989), which is essential for the transcription of essential early sporulation genes, including *spoOA* (Predich et al. 1992) and *spoIIA* (Wu et al. 1991). *SpoOA* is the key response regulator in the sporulation signal transduction pathway (Trach et al. 1992). Glucose, a catabolite inhibitor of sporulation, also represses *sinI* expression. *sinR* expression is unaffected by the genetic lesions and nutrient conditions described above (Gaur et al. 1986). This indicates that *sinR* is regulated differently than *sinI*. *sinR* is transcribed by the P3 promoter, which is downstream from *sinI*. Insertion of an antibiotic resistance cassette into *sinI* does not prevent *sinR* expression, which is in agreement with the above conclusion. Thus, it can be postulated that *sinI* acts as a mediator between stage O and stage II sporulation genes that are repressed by SinR and that the information flows from *spoOA* to *sinI*. During sporulation, SinR is down-regulated by elevated expression of *sinI*, and the formation of the SinI/SinR complex may be one of the steps necessary to initiate or maintain the sporulation process. Because strains containing a deletion of both *sinI* and *sinR* have a phenotype identical to those with only the *sinR* gene inactivated, we believe that the sole function of SinI is to modulate SinR function (U. Bai, I. Mandic-Mulec, and I. Smith, unpubl.).

What is the mechanism by which SinI represses SinR activity? SinR is a DNA-binding protein (Gaur et al. 1986). Its amino-terminal region, which contains a putative helix–turn–helix (HTH) domain at positions 17–36, has a high degree of similarity to the amino terminus of the bacteriophage ϕ 105R protein (Smith et al.). Extensive mutational analysis has shown that this region of a ϕ 105R contains a HTH DNA-binding domain (Van Kaer et al. 1988), and the similarity of the two proteins at their amino termini strongly suggests that this region is also the DNA-binding domain of SinR. Further analysis of the derived amino acid sequence of SinR revealed a series of leucine and other hydrophobic amino acids in heptad repeats following the putative DNA-binding domain, starting at position 47 and ending at position 68. These data suggested the presence of an amphipathic coil structure in this region, which was postulated to be the domain for SinR multimerization (Gaur et al. 1991). The speculative SinR structure is presented in schematic form in Figure 10A (top).

Data base analysis of SinI has revealed that of all the several thousand proteins screened, SinI shows the greatest similarity to SinR. As shown in Figure 10A, in a 36-amino-acid overlap which includes the carboxy-terminal region of SinR and most of the 57-codon *sinI* ORF, there is a 28% identity and an additional 20% conservation of amino acids. Thus, SinI is missing the postulated DNA-binding domain of SinR; as shown in Results, SinI does not bind to DNA. A possible model of SinI function is suggested by the amino acid similarity to the carboxy-terminal domain of SinR, its inability to shift DNA containing the *aprE* promoter, and its ability to form immunoprecipitable complexes with SinR. Because SinR, in solution, behaves as a tetramer, SinI may displace SinR monomers from SinR homomultimers to form hetero-

Figure 10. Proposed SinI/SinR interactions. (A) A schematic model of SinI and SinR, including proposed functional domains, is shown at the top. The derived amino acid sequences for residues 65–104 of SinR and the SinI amino acid sequence from residue 2 to 40 shown below were aligned according to data base analysis. The shaded boxes indicate identical or chemically equivalent amino acids (Zuber et al. 1989). (B) Two possible models for SinI/SinR interaction are illustrated. The functional SinR multimer, which exists predominantly as a tetramer in solution (Gaur et al. 1991), is shown (left) as a dimer, for clarity. The DNA-binding domain is at the base of the structure. (Center) SinI forming a heterodimer with a SinR monomer, which prevents SinR function. (Right) SinI is shown binding to the multimerization domain of the SinR functional dimer, causing an undefined change in structure, which prevents DNA binding.

multimers. These complexes may be incapable of binding to DNA targets. In this way SinI would act as a dominant-negative inhibitor of SinR function. Many systems in eukaryotic organisms exhibit this type of regulation. Id, a helix–loop–helix (HLH) protein, is missing the basic region adjacent to the HLH domain that is essential for the specific DNA binding in another HLH protein, MyoD (Benezra et al. 1990). Id associates with MyoD and prevents it from binding to DNA, as MyoD can no longer form homodimeric or heterodimeric complexes with other positively acting HLH proteins. I-POU, a POU domain protein in *Drosophila*, prevents the binding of the neuron-specific DNA regulatory POU protein Cf-1 to its target DNA by forming a stable heterodimer (Treacy et al. 1991). Because I-POU is lacking two essential amino acids in the basic region, it cannot bind to DNA. A last example is IP-1. Although its genetic determinant has not been sequenced yet, IP-1 inhibits the DNA-binding activity of AP-1 (Jun) in mammalian cells by binding to the LZ region of AP-1 (Auwerx and Sassone-Corsi 1991) and is believed to function in a manner similar to Id and I-POU. It is also possible that SinI interacts with a func-

tional SinR multimer to prevent its binding to DNA. This would be similar to the interaction of the *Salmonella* bacteriophage P22 antirepressor with the P22 and λ C1 repressors. The antirepressor prevents the two repressors from functioning in vivo (Susskind and Youderian 1983), and it binds to the multimerization domains of the functional repressor multimers in vitro. Unlike the SinI/SinR interaction, the association of antirepressor to λ C1 does not prevent its binding to DNA (R. Sauer, pers. comm.).

However, these theories of SinI/SinR interactions are purely speculative at this time. For example, the putative LZ region of SinR (amino acids 47–61) is not included in the area of similarity to SinI, which begins at amino acid 66. Therefore, evidence for the proposed model awaits the results of detailed mutational analysis of both proteins and SinR DNA target sites. In any case, the results presented here show that the novel interactions of two regulatory proteins not only influence cell growth and differentiation but may also allow for the coordination of different developmental pathways.

Interestingly, the activities of σ^B and σ^F , two σ -factors that also control late growth processes in *B. subtilis*, are regulated post-translationally by proteins that are coordinately produced. σ^F , the product of the *spoIIAC* gene, is negatively controlled by the product of the cotranscribed *spoIIAB* (Schmidt et al. 1990), and σ^B , the product of the *sigB* gene, is negatively modulated by the product of the cotranscribed *sigB-orfW* gene (Benson and Haldenwang 1992; Boylan et al. 1992). The nature of these interactions is not yet known but, as is the case with SinR/SinI, is presumably the result of direct protein–protein binding. Thus, it seems that protein–protein interaction is not an uncommon feature of prokaryotic gene regulation, as is true for eukaryotic systems.

Materials and methods

Plasmid and strain constructions

All bacterial strains are derivatives of IS75 (*leuA8 metB5 hisH2*) unless otherwise indicated. pIS229 is a multicopy plasmid containing the *sinR* gene and a phleomycin resistance marker (Mandic-Mulec et al. 1992). pIS253 is derived from pBD347 (Weinrauch et al. 1990), with its *MboI* site inactivated by mung bean nuclease treatment. The region of *sinI*, spanning from *XbaI* to *NruI*, was amplified by polymerase chain reaction (PCR) (Perkin-Elmer Cetus, Norwalk, CT) from plasmid DNA of pIS74 (Gaur et al. 1986), also adding *HindIII* sites to the ends of the DNA fragment. The amplified DNA was restricted with *HindIII* and cloned in pIS253, resulting in pIS256. This plasmid was then transformed into IS75 (Spo^+), selecting for Cm^r . One of the transformants with a rough phenotype was saved as IS1576. pIS257 was constructed from pIS256 by digesting this plasmid DNA at its unique *MboI*–*AvaII* sites. After filling-in and ligation reactions, this resulted in an in-frame deletion of 99 bp in the *sinI* ORF. This deletion was then transferred into the chromosome by gene conversion, in IS75, as it was predicted that disruption of the chromosomal *sinI* should give a sporulation-deficient phenotype. One of the sporulation-deficient colonies, having this in-frame deletion, was saved as IS874. The DNA sequence of *sinI* in IS874 was determined (using the Sequenase

version 2.0 DNA Sequencing Kit, U.S. Biochemical Corporation, Cleveland, OH) to ensure that the deletion was in-frame and no other mutation was picked up during amplification. For complementation experiments, the *HindIII* fragment of *sinI* in pIS256 was cloned into the integration vector pCP115 (Price and Doi 1985), resulting in pIS259. IS791 contains an integrated *sinI* behind a pSPAC promoter. To construct this strain, the region containing *sinI*, flanked by *HindIII* and *SalI*, was amplified by PCR and cloned into pDR66 (obtained from Alan Grossman, Massachusetts Institute of Technology, Cambridge). pDR66 contains the *lacI* gene and the pSPAC promoter in a plasmid that replicates in *E. coli* and has an ampicillin resistance (Amp^r) gene and a chloramphenicol resistance (Cm^r) gene that expresses in *B. subtilis*. This plasmid can integrate at the ectopic *amyE* locus. After ligation, the resulting plasmid was linearized at the unique *ScaI* site and was integrated into the *B. subtilis* chromosome at the *amyE* locus, selecting for Cm^r and giving strain IS791. IS803 contains a *comG*–*lacZ* fusion and a kanamycin resistance marker integrated at the *amyE* locus and was obtained from David Dubnau (Public Health Research Institute, NY). IS1593 and IS1594 were constructed by transforming IS803 with DNA from pIS256 and pIS257, respectively.

IS819 is a wild-type strain containing *spoIIA*–*lacZ* integrated into the chromosome. The *spoIIA*–*lacZ* fusion, which results from a Campbell-type insertion at the *spoIIA* locus, was described previously (Mandic-Mulec et al. 1992). IS820 was obtained by transforming IS819 with chromosomal DNA from IS791. IS879 was obtained by transforming IS874 with chromosomal DNA from IS819. IS657, used for protease assays, has *spoIIIG*–*lacZ* integrated into the homologous site in the chromosome. IS880 was obtained by transforming IS657 with chromosomal DNA from IS874. IS1491 was constructed by transforming pIS229 DNA into IS657.

Media and β -galactosidase determinations

All media [NSM, Luria broth (LB), minimal salts competence media] were described previously (Weinrauch et al. 1990). β -Galactosidase determinations were performed as described previously (Mandic-Mulec et al. 1992).

Photomicroscopy

Samples from isogenic *B. subtilis* cultures grown overnight at 32°C in NSM or treated with IPTG for pSPAC constructs were spotted on microscopic slides. These were observed and photographed in a Nikon Diaphot TMD-EF2 microscope with phase-contrast optics.

SDS-PAGE

Proteins were analyzed by electrophoresis in polyacrylamide gel containing SDS, as described by Laemmli (1970), followed by Coomassie staining.

Western blot analysis

Samples were subjected to electrophoresis as described above, and the proteins were electroblotted to a sheet of nitrocellulose transfer membrane (Millipore Corp., Bedford, MA). SinI antibodies were raised in guinea pigs against a synthetic polypeptide containing amino acids 12–47 of the SinI ORF. The SinR antibodies were raised in guinea pigs against purified SinR protein. Blotting and immunotechniques were performed as described previously (Bai et al. 1990).

Overproduction and purification of SinI

We cloned the *Hind*III fragment of pIS256, containing the *sinI* gene, in pT7-I (U.S. Biochemical) resulting in pIS281. After establishing this plasmid in *E. coli* BL21(DE3), a strain containing pLysS [Studier et al. 1990], these cells were grown in LB containing chloramphenicol (20 µg/ml) and ampicillin (100 µg/ml). When the culture reached OD₆₀₀ of 0.6–1.0, it was induced with 0.4 mM IPTG and grown for 2.5 hr. All subsequent steps were carried out at 4°C. The cells were harvested and broken in a French pressure cell in a buffer containing 10 mM Tris-HCl (pH 8.2), 10 mM MgCl₂, 1 mM EDTA, 0.3 mM dithiothreitol (DTT), 5% glycerol, and 1 mM PMSF, at 13,000 lb/in². The lysate was centrifuged at 38,000g for 40 min in a Sorvall centrifuge. An amount of the supernatant (2.5 ml) equivalent to 75 mg of protein was loaded onto a 8-ml DEAE-Sephacel (Sigma Chemical Co., St. Louis, MO) column, previously equilibrated with the same buffer. The column was washed with 40 ml of the buffer and eluted with 30 ml of 0–400 mM linear KCl gradient in the same buffer. The first few fractions contained almost pure SinI, detected by 12% SDS-PAGE and also by immunoanalysis as described above. Fractions containing SinI were pooled and stored at –20°C in aliquots.

Binding assays

The binding of SinR to *aprE* was performed using the 3' end-labeled 250-bp *Not*I–*Dde*I promoter fragment as described previously [Gaur et al. 1991]. To test the effect of SinI on SinR binding to *aprE*, SinI and SinR were mixed and incubated for 15 min at room temperature, before adding the DNA probe. Incubation in the presence of DNA was continued for 15 min more, and the products were separated on a 5% nondenaturing polyacrylamide gel at room temperature.

Immunoprecipitation experiments

Immunoprecipitation experiments were performed as described elsewhere [Bengal et al. 1992], with the modification presented below. [³⁵S]Methionine-labeled SinI was synthesized in vitro using an *E. coli* S-30 lysate (Amersham), supplemented with T₇ RNA polymerase, under conditions suggested by the manufacturer. Lysate samples containing labeled SinI were incubated with purified Sin protein at room temperature for 30 min in a buffer containing 20 mM HEPES-KOH (pH 7.9), 50 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, and 10% glycerol. The SinI/SinR complexes were then diluted to 200 µl with RIPA buffer, which contained 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, and 1 mM PMSF. SinR antibodies raised against purified SinR protein were then added. After overnight incubation at 4°C, protein A-Sepharose beads (40 µl) in RIPA buffer were added to the immune complexes, and they were placed in a rotary shaker for 2 hr at 4°C. After washing three times with RIPA buffer, the immune complexes were reduced by boiling in 2× SDS-PAGE sample buffer; they were then analyzed by SDS-PAGE, followed by autoradiography. For coimmunoprecipitation studies with nonradioactive proteins, 2 µg of purified SinR was mixed with 4 µg of purified SinI and incubated at room temperature for 30 min. Immunoprecipitation with SinI-specific antisera was done as described for SinR antisera above. The immunocomplex was electrophoresed on a 15% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with SinR-specific antisera, as described previously [Bai et al. 1990].

Protease assays

The protease activity in culture supernatants was assayed as described previously [Gaur et al. 1986]. Azocoll (25 mg) (Sigma

Chemical Co.) was added to suitable samples of cell supernatants in 0.1 M Tris-hydrochloride (pH 7.5) and water to give a final volume of 5 ml. The reaction mixture was incubated at 37°C for 10 min with shaking. The digests were then filtered through Whatman filter paper, and the OD₅₂₀ was measured in the filtrates. The blank was prepared the same way except that enzyme-free medium was added. Activity was defined as the change in OD₅₂₀ by 1 ml of culture supernatant at 37°C in 10 min (one optical density unit is equivalent to 1 unit of activity). The specific activity was obtained by dividing the activity by the corresponding number of Klett units of the culture.

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Note added in proof

Another example of protein–protein interaction down-regulating the activity of a prokaryotic DNA-binding protein is found in *E. coli* bacteriophage P1. The Cui protein is an antagonist of C1 repressor function in vivo and in vitro [T. Heinzl et al. (1990) *J. Biol. Chem.* **265**: 17928–17934].

The sequence data described in this paper have been submitted to the EMBL/GenBank data libraries under accession number M14112.

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