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FlgM Is a Primary Regulator of σ^D Activity, and Its Absence Restores Motility to a *sinR* Mutant

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We have used mini-Tn*10* mutagenesis to identify negative regulators of σ^D activity. Nine independent insertions were mapped to five genes: *flgM*, *flgK*, *fliD*, *fliS*, and *fliT*, suggesting that FlgM export is regulated similarly in *Bacillus subtilis* and *Salmonella typhimurium*. We show that a deletion of *flgM* can restore σ^D activity to a *sinR* null mutant of *B. subtilis*, although *fla/che* operon expression is affected by neither SinR nor FlgM.

About 50 genes are involved in making *Salmonella typhimurium* chemotactic, and expression of these genes is regulated in a hierarchical fashion (for reviews, see references 7 and 16). Late gene expression is dependent on the flagellar sigma factor, σ^{28}, whose activity is negatively regulated by FlgM (5, 13). FlgM acts as an anti-sigma factor, binding to and inactivating σ^{28} intracellularly until a functional hook basal body (HBB) is assembled (9, 12, 22). At that point, FlgM is actively exported through the HBB, thereby freeing σ^{28} to direct transcription of late flagellar genes (8, 11).

A similar regulatory hierarchy exists among the genes involved in *Bacillus subtilis* motility (1, 2). However, the σ^{28} factor, σ^D, directs transcription of not only late flagellar genes but also *degR* and genes involved in cell wall turnover (10, 14, 17, 21, 23). In addition, SinR, a pleiotropic regulator involved in sporulation, competence, and protease production, positively regulates *B. subtilis* motility (24, 26). These novel aspects of *B. subtilis* motility development suggested to us that σ^D may be uniquely regulated. The strains used in this study are listed in Table 1.

Mini-Tn*10* insertion mutations causing increased σ^D activity. To identify negative regulators of σ^D, we selected mutants with elevated σ^D activity. The σ^D-dependent *hag* promoter contains an upstream promoter (UP) element, between positions −42 and −65, which stimulates transcription more than 20-fold (4). We cloned the minimal *hag* promoter upstream of a *cat-lacZ* transcriptional fusion in plasmid pJPM122 (25), designed for recombination with a SPβ*c2*Δ2::Tn*917*::pSK10Δ2 prophage (30). Lysogens carrying this fusion appear white on plates containing 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. Transducing lysate {SPβ*c2*Δ2::Tn*917*::φ[*hag*(−UP)-*cat-lacZ*] *neo*}, without a UP element (−UP), was incubated with several independent mini-Tn*10* mutagenized cell libraries constructed with plasmid pIC333 (28), and transductants were spread onto plates of rich medium X-Gal containing 100 μg of spectinomycin per ml, 8 μg of neomycin per ml, and 5 μg of chloramphenicol per ml to select for the mini-Tn*10*, the SPβ prophage, and increased expression of the *hag*(−UP)-*cat-lacZ* fusion, respectively. At 5 μg of chloramphenicol per ml, a high background of light blue colonies was observed after 48 h at 37°C (2 to 10% of the estimated transductants plated). Within this background, a few dark blue colonies appeared. Only those mutants that exhibited high σ^D activity genetically linked to the mini-Tn*10* were further characterized.

Nine independent mini-Tn*10* insertions which increased expression from the minimal *hag* promoter were obtained (Fig. 1 and Table 2). Three insertions were at an identical site in *flgM*, a gene shown previously to negatively regulate σ^D activity (20).

TABLE 1. *B. subtilis* strains used in this study

Strain	Characteristic(s)	Reference or source
ZB307A	JH642 SPβ*c2*Δ2::Tn*917*::pSK10Δ6 (*trp*$^+$ *phe*$^+$)	25
HB1000	ZB307A *att*SPβ	This work
CB149	JH642 *flgM*Δ80	20
CU1065	*trpC2 att*SPβ	S. Zahler
OI2141	OI1085 Tn*917*Ω1836 (class 2 mutation)	31
OI2139	OI1085 Tn*917*Ω1832 (*fla/che'-lacZ*)	31
HB4097	CU1065 SPβ*c2*Δ2::Tn917::φ[P_{hag}(−UP)-*cat-lacZ*] *neo*	This work
HB4197	CB149 *sinR*::*neo*	This work
HB4204	HB1000 *sinR*::*neo*	This work
HB4207	HB1000::pHAG-96a [P_{hag}(+UP)-*lacZ* at *pks* locus]	This work
HB4208	CB149::pHAG-96a	This work
HB4209	HB1000::pHAG-41 [P_{hag}(−UP)-*lacZ* at *pks* locus]	This work
HB4210	CB149::pHAG-41	This work
HB4211	HB1000::pFLID-224 (P_{fliD}-*lacZ* at *pks* locus)	This work
HB4212	CB149::pFLID-224	This work
HB4213	HB4207 *sinR*::*neo*	This work
HB4214	HB4209 *sinR*::*neo*	This work
HB4215	HB4210 *sinR*::*neo*	This work
HB4216	HB4208 *sinR*::*neo*	This work
HB4217	HB4211 *flgM*::mini-Tn*10*	This work
HB4218	HB4211 *flgK*::mini-Tn*10*	This work
HB4219	HB4211 *fliD*::mini-Tn*10* (C43 allele)	This work
HB4220	HB4211 *fliS*::mini-Tn*10*	This work
HB4221	HB4211 *fliT*::mini-Tn*10*	This work
HB4222	HB4211 Tn*917*Ω1836	This work
HB4223	HB4212 Tn*917*Ω1836	This work
HB4224	HB4217 Tn*917*Ω1836	This work
HB4225	HB4218 Tn*917*Ω1836	This work
HB4226	HB4219 Tn*917*Ω1836	This work
HB4227	HB4220 Tn*917*Ω1836	This work
HB4228	HB4221 Tn*917*Ω1836	This work
HB4229	HB1000 *flgM*::mini-Tn*10*	This work
HB4230	HB1000 *flgK*::mini-Tn*10*	This work
HB4231	HB1000 *fliD*::mini-Tn*10* (C43 allele)	This work
HB4232	HB1000 *fliS*::mini-Tn*10*	This work
HB4233	HB1000 *fliT*::mini-Tn*10*	This work
HB4235	HB1000 Tn917Ω1832 (*fla/che'-lacZ*)	This work
HB4238	HB4235 *sinR*::*neo*	This work
HB4240	HB4235 *flgM*::mini-Tn*10*	This work

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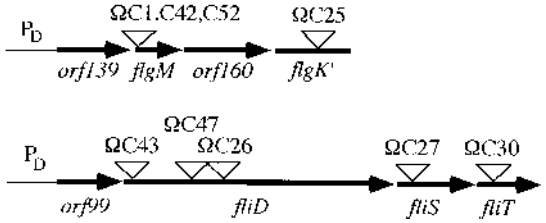

FIG. 1. Schematic representation of mini-Tn*10* insertions obtained in this study, all of which map to genes in *flgMK* (20) and *fliDST* (3) operons. Exact insertion locations are listed in Table 2. Transcription of both operons is directed by a σ^D-dependent promoter (P_D).

Each *flgM*::mini-Tn*10* strain was isolated from an independent cell library, and thus these strains are not siblings. Interestingly, the site of these insertions matches the *Escherichia coli* Tn*10* "hot spot" consensus sequence at only two of six positions (6, 15). The other six insertions were located in *flgK*, which is likely cotranscribed downstream from *flgM*, and the *fliDST* operon (Fig. 1).

To quantitate the effects of these gene disruptions on σ^D activity, each mutation was crossed into a strain containing a P_{fliD}-*lacZ* reporter fusion (at *pks*), and β-galactosidase was assayed during growth in Difco sporulation medium. The *fliD* promoter, like the *hag* promoter, is exclusively σ^D dependent (3). An insertion in *flgM* elevates expression of the *fliD* promoter 15-fold, similar to the effect of an in-frame deletion of *flgM* (*flgM*Δ80) on P_{hag} (lacking the UP element) expression (see Fig. 3). Insertions in *fliD*, *fliS*, and *fliT* increase P_{fliD}-*lacZ* expression 5-, 6-, and 1.5-fold, respectively. An effect of the *flgK*::mini-Tn*10* insertion on σ^D expression in liquid culture was not detectable despite an obvious effect on plates.

Mutations, designated class 2, which prevent the assembly of a complete HBB, block σ^D activity but only modestly affect σ^D expression (18, 20). One such mutation, Tn*917*Ω1836, disrupts the major *fla*/*che* operon near *fliF* (31) and confers a nonmotile and filamentous phenotype. We quantitated the effects of Tn*917*Ω1836 on P_{fliD} expression in wild-type, *flgM*Δ80, and various mini-Tn*10* insertion backgrounds. Only mutations in *flgM* restore P_{fliD} expression and proper cell separation in this class 2 background. The failure of the other mutations to increase σ^D activity in a class 2 background is consistent with the idea that they act by increasing FlgM export and therefore require a functional HBB.

Each mini-Tn*10* was crossed into wild-type HB1000, and the resulting strains were spotted onto plates containing mannitol to assay motility (Table 2). Mutants containing insertions in *flgK*, *fliD*, *fliS*, and *fliT* were completely nonmotile by this assay, consistent with previous data concerning *fliD*, *fliS*, and *fliT* (3). The *flgM*::mini-Tn*10* strain had a reduced rate of motility (30% of that of the wild type). This phenotype is likely due to partial polarity on genes downstream of *flgM*, since strains harboring an in-frame deletion of *flgM* have an increased rate of motility (Table 2 and Fig. 2).

Deletion of *flgM* suppresses two phenotypes of a *sinR*::*neo* strain by restoring σ^D activity. SinR negatively affects sporulation and protease production but positively affects competence, motility, and autolysin genes (26). Mutants of *sinR* are nonmotile and grow filamentously, as do *sigD* mutants. The reduced σ^D activity in *sinR* strains may explain the filamentous and nonmotile phenotypes conferred by *sinR* mutations (1, 3, 19). To test whether the *flgM*Δ80 mutation can restore σ^D activity to the *sinR* null mutant, we constructed the double null mutant and examined it by phase-contrast light microscopy. Indeed, the *flgM*Δ80 mutation restores both motility (Fig. 2) and proper cell separation to the *sinR*::*neo*-containing strain. In contrast, the rough-colony phenotype conferred by the *sinR*::*neo* mutation was not altered by *flgM*Δ80.

To quantitate the effects of the *sinR*::*neo* and *flgM*Δ80 mutations on σ^D activity, we used *hag* promoter fusions containing or lacking the UP element (Fig. 3). As expected, σ^D activity increased in the *flgM*Δ80 background, though this effect was most dramatic (13-fold) in the construct lacking the UP element. σ^D activity was decreased by the *sinR*::*neo* mutation, especially before time zero, but the *sinR* effect on maximal *hag* expression was relatively modest (two- to threefold). Surprisingly, the *sinR flgM* double null mutant showed maximal levels of expression higher than those of both the wild-type and *flgM*Δ80 strains. Thus, σ^D activity is restored to high levels in a *sinR* null strain by a secondary *flgM* null mutation.

Null mutations in *sinR* or *flgM* do not affect expression of the major *fla*/*che* operon. The *sigD* gene is near the end of the >25-kb major *fla*/*che* operon (18). To determine the effect of the *sinR*::*neo* and *flgM*::mini-Tn*10* mutations on *fla*/*che* operon expression, we used Tn*917*Ω1832*lacZ*, located about 6.5 kb downstream from the *fla*/*che* promoter region (31). This insertion is a class 2 mutation: strains carrying Ω1832 are nonmotile but still express the σ^D protein (18). A *flgM*::mini-Tn*10* insertion did not affect Tn*917*Ω1832*lacZ* expression in cells grown in Difco sporulation medium, suggesting that σ^D does not contribute significantly to *fla*/*che* expression under these conditions. The *sinR*::*neo* mutation also did not affect *fla*/*che* expression in cells harboring Tn*917*Ω1832*lacZ*. Consistent with

TABLE 2. Mutations analyzed in this study

Mutation designation	% Motility (mean ± SEM)[a]	Gene disrupted	Codon disrupted	Site of insertion[b]	Sequence reference
*flgM*Δ80	197 ± 2	*flgM*	NA[c]	NA	20
C1, C42, C52	30 ± 9	*flgM*	10th	1348-aacacaatc-1356	20
C25	0	*flgK*	68th	2304-gtacgggcg-2312	20
C26	ND[d]	*fliD*	177th	1197-tacagagta-1205	3
C43	0	*fliD*	45th	800-agcgtgaca-808	3
C47	ND	*fliD*	125th or 129th	1043-acaatttgg-1051 or 1052-catttaacg-1060	3
C27	0	*fliS*	30th	2273-tgtataatg-2281	3
C30	0	*fliT*	37th	2699-tacacggtc-2707	3

[a] Wild-type HB1000 swims at 0.74 mm/h at 37°C through these plates. The results are expressed as the percentage of the wild-type rate of motility. The standard error of the mean equals $\sigma_{n-1}/n^{1/2}$.
[b] The sequences listed represent the 9 bp duplicated by the transposition event and the position numbers.
[c] NA, not applicable.
[d] ND, not determined.

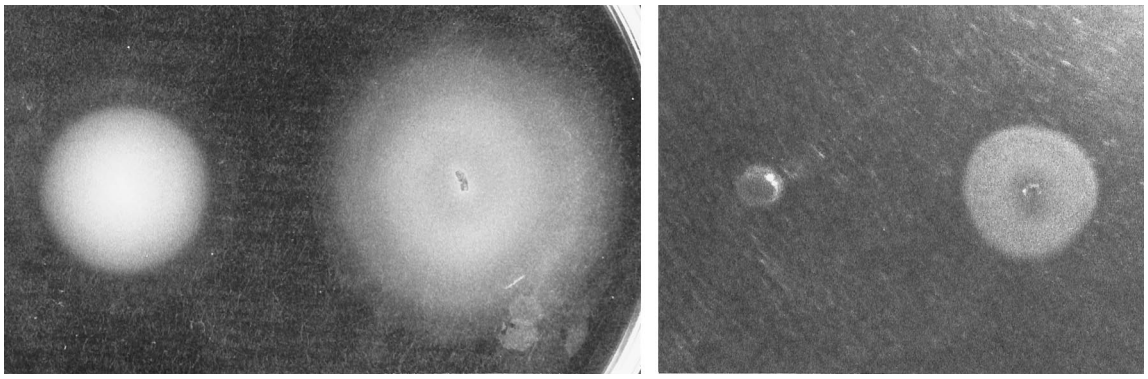

FIG. 2. Motility analysis. Cells from single colonies cultured overnight were inoculated onto plates containing 0.4% agar-tryptone and incubated at 37°C for 14 h. HB1000 (wild type), CB149 (*flgM*Δ80), HB4204 (*sinR*::*neo*), and HB4197 (*flgM*Δ80 *sinR*::*neo*) are shown (from left to right).

this, Smith found no effect of *sinR* disruptions on *sigD'-lacZ* expression or several other *lacZ* gene fusions within the *fla*/*che* operon (27). These results suggest that SinR affects σ^D activity rather than *sigD* expression.

Conclusions. We have shown that mini-Tn*10* insertions in *flgM*, *flgK*, *fliD*, *fliS*, and *fliT* increase the activity of σ^D in *B. subtilis*. By analogy with recent work on the corresponding *S. typhimurium* mutants (29), we suggest that insertions in *fliD*, *fliS*, and *fliT* indirectly affect σ^D activity by increasing FlgM export. The insertion in *flgK* might also affect FlgM export. Alternatively, *flgK*::mini-Tn*10* could affect the synthesis of FlgM, since *flgM* and *flgK* are thought to be cotranscribed in *B. subtilis* (20). Thus, FlgM seems to be the only dispensable protein in *B. subtilis* which negatively regulates σ^D directly. SinR is also required for motility in *B. subtilis*, and the effects of one *sinR* mutation on *sigD* expression suggested that this might be due to a role of SinR in activating expression of the major *fla*/*che* operon (10). However, our results together with those of Smith (27) indicate that the SinR requirement for motility can be bypassed by deletion of *flgM* and that SinR is not necessary for σ^D expression.

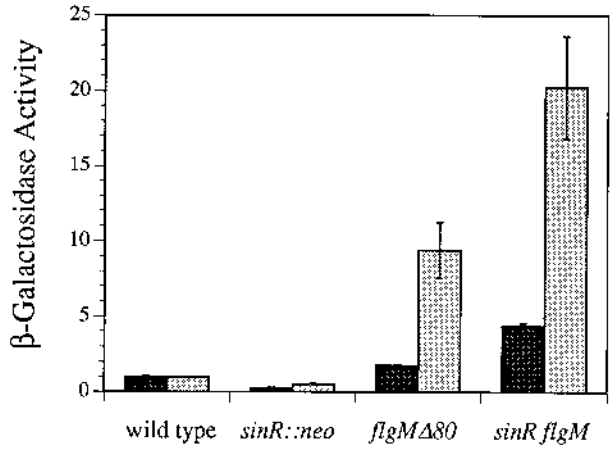

FIG. 3. Maximal β-galactosidase activity in wild-type and mutant strains grown in Difco sporulation medium harboring P_{hag}(+UP)-*lacZ* (dark bars) and P_{hag}(−UP)-*lacZ* (light bars) reporter fusions. Results are normalized to those for the wild-type strains, in which the P_{hag}(+UP)-*lacZ* strain produces 1,590 Miller units and the P_{hag}(−UP)-*lacZ* strain produces 20 Miller units. Error bars represent standard errors of the means, which equal $\sigma_{n-1}/n^{1/2}$ with $n = 3$.

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