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Genetic Control of Flagellation in *Bacillus subtilis*

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Flagellation in *Bacillus subtilis* was shown to involve at least three loci: a gene *H* controlling the specificity of the flagellar antigen; a gene *fla* controlling the presence or absence of flagella; and a gene *mot* controlling the function of the flagella. The loci were shown to be nonallelic and unlinked in transformation tests. Strains W 23 and SB 108-b, a derivative of strain 168, were shown to differ in their major flagellar antigen.

Bacterial flagella consist of aggregates of protein molecules termed "flagellins," a given flagellum containing only one species of flagellin molecule (1). In *Salmonella*, phase variation has been described, in which two flagellar antigens specified by separate, unlinked genes are alternately produced (7). These genes were termed *H* genes and were later defined as the structural genes for the alternate flagellin molecules (8). Mutation in the *H* genes produced altered flagellins which have been recognized antigenically (10, 12) and functionally (6, 10). Other genes affecting flagellar characters have been recognized in *Salmonella* (8), of which two classes were well characterized: *fla* genes, controlling the presence or absence of flagella, and *mot* genes, affecting the function of the flagella when formed. Phase variation was shown to involve a complex system of regulation (15).

The flagella of various species of *Bacillus* have been shown to be biochemically similar to those of *Salmonella* (1). However, no investigation of the genetic control of flagellation in this genus has been reported. Preliminary experiments are described which show that in *B. subtilis* the genetic control of flagellation is similar to that described for *Salmonella*, with the exception that no antigenic phase variation could be detected.

MATERIALS AND METHODS

Strains. The strains of *B. subtilis* used and prepared in the investigation are shown in Table 1. Genetic markers are symbolized in accordance with recent recommendations (4). The symbol *H* is used for the gene determining the antigenic specificity of the flagellum, with the antigenic factor it specifies indicated after a hyphen; e.g., *H-a* is used as the symbol for the gene determining the flagellar antigenic factor *a*. For the isolation of nonmotile mutants, cultures were exposed to ultraviolet irradiation, and individual clones of the surviving cells were scored for motility in semisolid medium (5).

Transformation. The methods described by Anagnostopoulos and Spizizen (2) were used for the isolation of transforming deoxyribonucleic acid (DNA), the preparation of competent cells, and the assay of transformation involving biochemical markers. For quantitative transformation of motility markers, a modification of Stocker's method (19) was used. Competent cultures, after exposure to DNA, were diluted, and 10 standard loopfuls of each dilution were individually inoculated to semisolid medium (9) contained in 1-ml volumes in cups of sterile plastic hemagglutination trays. At dilutions at which about half of the inoculated loopfuls gave rise to swarms (20), each swarm was assumed to have arisen from one original motile transformant, and the data were used to calculate the number of cells transformed to motility. To select for transformants with the specific flagellar antigen of the donor, sterile rabbit anti-flagella serum prepared against the recipient was incorporated into the semisolid medium (20). The dilution of the antiserum in this procedure prevented effects of antibodies directed against the somatic antigens. In all cases, reconstruction experiments were performed to check the efficiency of the serum-semisolid selection media. Linkage tests were made by the method of Nester et al. (16). Double transformants produced solely at saturating concentrations of DNA were assumed to result from double infection with different DNA molecules, each carrying only one of the transformed genes. For evidence of linkage, quantitative measurements of double and single transformants at nonsaturating DNA concentrations were used to calculate values for the co-transfer index (16).

Serological methods. Methods previously described for the serological analysis of mutant forms of the *i* antigen of *S. typhimurium* (12) were adapted for use with *B. subtilis*. Motile strains were grown in Penassay Broth (Difco) to late log phase (10^8 viable cells/ml) and killed by the addition of 0.3% (v/v; final concentration) Formalin. Such suspensions were used to immunize rabbits and as agglutinable suspensions in titrations of the antisera obtained. All serum titrations described were performed by a micro-method (12). Absorption of an antiserum with flagellated

TABLE 1. *Strains of Bacillus subtilis*

Strain	Derivation	Mutant loci and mutation sites							Relevant phenotype[a]					
		trp-2	*his-2*	*H*	*fla-1*	*fla-6*	*fla-48*	*mot*	*str*	Trp	His	Antig	Flag	Paral	Str
W 23	W 23 wild-type (3)	+	+	*H-b*	+	+	+	+	+	+	+	*b*	+	−	s
SB 108	*fla*⁻ derivative of 168 (19)	−	+	*H-a*	−	+	+	+	+	−	+	(*a*)	−	(−)	s
SB 108-b	*fla*⁺ mutant of SB 108 (9)	−	+	*H-a*	+	+	+	+	+	−	+	*a*	+	−	s
MS 10	Derivative of 168 (9)	−	−	*H-a*	+	+	+	+	−	−	−	*a*	+	−	r
SO 5	Paralyzed mutant of SB 108-b (5)	−	+	*H-a*	+	+	+	−	+	−	+	*a*	+	+	s
SO 6	*fla*⁻ mutant of SB 108-b (5)	−	+	*H-a*	+	−	+	+	+	−	+	(*a*)	−	(−)	s
SO 12	W 23 −× SB 108-b[b]	−	+	*H-b*	+	+	+	+	+	−	+	*b*	+	−	s
SO 14	MS 10 −× SB 108-b	−	+	*H-a*	+	+	+	+	−	−	+	*a*	+	−	r
SO 49	SO 5 −× SO 14	−	+	*H-a*	+	+	+	−	−	−	+	*a*	+	+	r
SO 55	SO 5 −× SO 12	−	+	*H-b*	+	+	+	−	+	−	+	*b*	+	+	s
SO 65	*fla*⁻ mutant of W 23	+	+	*H-b*	+	+	−	+	+	+	+	(*b*)	−	(−)	s
SO 67	SB 108-b −× W 23	+	+	*H-a*	+	+	+	+	+	+	+	*a*	+	−	s

[a] Trp, tryptophan; His, histidine; Antig, flagellar antigen; Flag, flagellated; Paral, paralyzed; Str, streptomycin; r, resistant; s, susceptible.

[b] The symbol W 23 −× SB 108-b indicates that DNA from strain W 23 was used to transform strain SB 108-b (16).

derivatives of the strain used to prepare it resulted in complete removal of agglutinating activity against the immunizing strain, provided the *H* locus was not deliberately changed, indicating the stability of the flagellar antigens in *B. subtilis*.

RESULTS

Serological analysis of strains W 23 and SB 108-b of B. subtilis. The results of a serological analysis of antisera prepared against strains W 23 and SB 108-b, a derivative of strain 168, are shown in Table 2. Titration of anti-W 23 and anti-SB 108-b sera on agglutinable suspensions of both flagellated and nonflagellated strains demonstrated the expected existence of flagellar and somatic antigens, confirmed in each case by absorption with the homologous nonflagellated strain. Each serum agglutinated flagellated and nonflagellated suspensions of the heterologous strain to the same end point. This indicates the existence of a common somatic antigen in W 23 and SB 108-b. Removal of activity against the common somatic antigen, by absorption with the heterologous nonflagellated strain, was found to reveal a low-titer activity against the heterologous motile strain. This indicates the presence of a minor common flagellar antigenic factor in the two strains. By analogy with the Kauffmann-White scheme for *Salmonella* (13), these results can be represented as:

B. subtilis SB 108-b = 1,2 :*a*. .
B. subtilis W 23 = 1,3 :*b*. .

where 1, 2, and 3 represent somatic antigens and *a* and *b* represent flagellar antigens (with minor components indicated as . .). These results were confirmed by use of antigenically hybrid strains prepared by transformation: SO 12 = 1,2 :*b* and SO 67 = 1,3 :*a*.

There is no description in the literature of phase variation in *B. subtilis*. If strain W 23 and SB 108-b were fixed in alternate phases of a phase-variation system, then each would produce flagella serologically different from the other; such a system has been reported for certain strains of *Salmonella* (7), in which the alternate phase was isolated by serum selection in semisolid medium. Growth of W 23 in semisolid medium containing anti-*b* serum, and of SB 108-b in semisolid medium containing anti-*a* serum, failed to produce strains with the alternate flagellar antigen.

Identification of the H gene. The demonstration of distinct flagellar antigens in strains W 23 and SB 108-b provided what were presumably allelic forms of a gene controlling this specificity. Using

TABLE 2. *Serological analysis of Bacillus subtilis strains W 23 and SB 108-b*

Serum	Absorbed with	Dilution in absorption	Log titer of absorbed sera on[a]						
			SB 108-b (*fla*$^+$)	SB 108 (SB 108-b *fla*$^-$)	SO 6 (SB 108-b *fla*$^-$)	W 23 (*fla*$^+$)	SO 65 (W 23 *fla*$^-$)	SO 12 (SB 108-b *fla*$^+$ *H-b*)	SO 67 (W 23 *fla*$^+$ *H-a*)
Anti-W 23	0	0	4	4	4	14	10	14	10
	SO 65 (W 23 *fla*$^-$)	1:12	3	—	—	14	—	14	3
	SB 108 (SB 108-b *fla*$^-$)	1:6	3	—	—	14	10	14	10
	SO 12 (SB 108-b *fla*$^+$ *H-b*)	1:12	—	—	—	9	9	—	9
	SO 12 and W 23	1:24	—	—	—	—	—	—	—
	SO 12 and SO 65	1:24	—	—	—	—	—	—	—
	SO 67 (W 23 *fla*$^+$ *H-a*)	1:12	—	—	—	14	—	14	—
Anti-SB 108-b	0	0	12	10	10	8	8	10	12
	SO 65 (W23 *fla*$^-$)	1:6	12	10	10	3	—	10	12
	SB 108 (SB 108-b *fla*$^-$)	1:24	12	—	—	3	—	3	12
	SO 12 (SB 108-b *fla*$^+$ *H-b*)	1:12	12	—	—	—	—	—	12
	SO 67 (W 23 *fla*$^+$ *H-a*)	1:12	10	10	10	—	—	10	—

[a] Antisera, before and after complete absorption, were tested by the micromethod. Their titers are stated as the last effective dilution, expressed as log to base 2, in terms of the unabsorbed sera (obtained by calculation, with use of the dilution involved in absorption).

serum selection in semisolid medium, we found that transformants with the *a* antigen could be produced by exposing competent cells of W 23 (*fla*$^+$) to DNA isolated from SB 108-b (*fla*$^+$); SB 108-b transformants with the *b* antigen could be produced by the reverse cross. Quantitatively, SB 108-b yielded the same number of transformants with the *b* flagellar antigen as transformants with the *trp-2*$^+$ marker when exposed in the competent state to DNA from W 23 at concentrations below saturation. Absorption tests (Table 2) demonstrated that, in both directions of the cross, the major flagellar antigen of the recipient strain was replaced by that of the donor, but the involvement of the minor common antigen could not be ascertained; somatic antigens were unchanged in the transformants.

As *B. subtilis* did not appear to undergo phase variation in its flagellar antigens, the simplest explanation of these results was that they demonstrated the existence of a locus, called *H* by analogy with the nomenclature used in *Salmonella* (8), which controlled the flagellar antigen; SB 108-b possessed the *H-a* allele and W 23 the *H-b* allele.

Identification of a fla gene. It has been demonstrated (19) that nonflagellated strains of *B. subtilis* may be obtained and that they can be transformed to motility by DNA isolated from a flagellated strain. Such mutants have been termed *fla*$^-$ mutants. In confirmation of these results, the nonflagellated mutant SO 6, derived from SB 108-b, was transformed to motility with DNA from either strain W 23 or SB 108-b. With W 23 DNA, SO 6 was transformed to motility at the same frequency as it was transformed to *trp-2*$^+$.

Identification of a mot gene. A mutant strain of *B. subtilis* SB 108-b has been described (5) which is nonmotile in liquid and semisolid media even though it possesses flagella, demonstrable both by agglutination with specific antiserum and by staining. This paralyzed strain, SO 5, was found to be transformed to motility when exposed to DNA from either W 23 or SB 108-b. The mutation which caused this paralysis of flagella is described as being in a *mot* gene (4).

Mutations in *Salmonella* have been described which result in the production of flagella having half the normal wavelength and a marked inability to function as organs of locomotion. Such mutations have been termed "curly" and shown to be inseparable from the *H* locus (6). SO 12, SB 108-b given the H antigen *b* of W 23 by transformation, transformed with DNA from the SB 108-b *mot*$^-$ mutant SO 5, yielded transformants possessing paralyzed flagella with the *b* antigen unchanged. This indicates separation of the *H* and *mot* genes in *B. subtilis* and demonstrates that the *mot*$^-$ mutation of SO 5 is a true *mot* mutation and not a curly mutation.

Test for linkage of the H and fla-6 loci. As described above, exposure of competent cells of SO 6 (SB 108-b *fla-6*$^-$) to DNA extracted from W 23 resulted in the production of motile transformants which were isolated by selection in semisolid medium. The addition of anti-SB 108-b serum to the

semisolid medium allowed the selection of double transformants which were transformed for both *fla*$^+$ and *H-b*. Double transformants were isolated only when the cells were concentrated prior to inoculation and the W 23 DNA was used at saturating concentrations, which indicated that the *H* and *fla* loci were unlinked with DNA isolated as described (2).

Test for linkage of the H and mot loci. Linkage between the *H* and *mot* loci was tested by use of SB 108-b *mot*$^-$ (SO 5 or SO 49) as recipient and DNA extracted from W 23, with selection for *mot*$^+$ transformants in semisolid medium and for *mot*$^+$ *H-b* transformants in semisolid medium containing anti-SB 108-b serum. Double transformants were isolated only when the DNA was used at saturating concentrations, which indicated the absence of linkage between the *H* and *mot* loci.

Test for linkage of the fla-6 and mot loci. Linkage between these two loci could not be tested directly, as mutation in either resulted in nonmotility. However, using saturating concentrations of DNA, we found that SO 6 (*fla-6*$^-$) yielded the same number of *fla*$^+$ transformants with DNA from SO 5 (*mot*$^-$) as with DNA from SB 108-b (*mot*$^+$) and, similarly, SO 5 (*mot*$^-$) yielded the same number of *mot*$^+$ transformants with DNA from SO 6 (*fla-6*$^-$) and SB 108-b (*fla*$^+$). In cases of close linkage (see reference 16 for the linked *trp-2 his-2* loci), the majority of transformants undergo a recombinational event involving both linked genes. If such close linkage existed here, DNA from a *mot*$^-$ *fla-6*$^+$ donor would yield fewer motile (*mot*$^+$ *fla*$^+$) transformants from a *mot*$^+$ *fla-6*$^-$ recipient than would DNA from a *mot*$^+$ *fla*$^+$ donor, because the majority of the *fla*$^+$ transformants would incorporate also the *mot*$^-$ gene of the donor; the reverse cross would be similar. As such a reduction was not observed experimentally, it was concluded that the *mot* and *fla-6* were not closely linked.

Tests for linkage of the three flagellar genes to the trp-2 gene. Competent cells of SO 5 (SB 108-b *mot*$^-$) and SO 6 (SB 108-b *fla-6*$^-$) were exposed to DNA from W 23, and transformants were selected. Motile transformants were tested for tryptophan requirement, and *trp-2*$^+$ transformants were tested for motility. The results are shown in Tables 3 and 4, from which it may be seen that double transformants of the *trp-2*$^+$ *mot*$^+$ and the *trp-2*$^+$ *fla-6*$^+$ types occurred at low levels and gave *r* values (co-transfer index, see reference 18) typical of unlinked markers. Double transformants of the *trp-2*$^+$ *H-b* type were extremely rare and did not indicate linkage of the two markers.

Transformation with nonmotility markers used as donors. Competent cells of SO 14, an SB 108-b *fla*$^+$ *mot*$^+$ derivative, were exposed in separate experiments to DNA from SO 6 (*fla-6*$^-$) and DNA from SO 5 (*mot*$^-$) and, after 30 min of exposure, were plated for single colonies. In each case, about 1,000 of the resulting colonies were tested for motility by inoculation of complete colonies into semisolid medium; nonmotile colonies detected were tested with the DNA used in their preparation to ensure genetic identity of their

TABLE 3. *Transformation of strain SO 6 (SB 108-b fla 6*$^-$*) with DNA from strain W 23*

Dilution of DNA used	Swarms in media containing anti-SB 108-b serum	Per cent transformed for *trp*$^+$	Per cent transformed for *fla*$^+$	Per cent of *trp*$^+$ transformants also transformed for *fla*$^+$	Per cent of *fla*$^+$ transformants also transformed for *trp*$^+$	*r* values for *fla-6* and *trp-2*
10^{-1}	0	0.05	0.04	Not counted	Not counted	—
10^{-2}	0	0.04	0.03	Not counted	Not counted	—
10^{-3}	0	0.004	0.004	Not counted	Not counted	—
10^{-4}	0	0.0005	0.0006	2	2	0.01

TABLE 4. *Transformation of strain SO 49 (SB 108-b mot*$^-$*) with DNA from strain W 23*

Dilution of DNA used	Swarms in media containing anti-SB 108-b serum	Per cent transformed for *trp*$^+$	Per cent transformed for *mot*$^+$	Per cent of *trp*$^+$ transformants also transformed for *mot*$^+$	Per cent of *mot*$^+$ transformants also transformed for *trp*$^+$	*r* values for *mot* and *trp-2*
Undiluted	Few	—	—	—	—	—
10^{-1}	0	0.17	0.07	Not counted	Not counted	—
10^{-2}	0	0.10	0.05	Not counted	Not counted	—
10^{-3}	0	0.015	0.020	1	1	0.01
10^{-4}	0	0.0013	0.0015	0	0	0

fla (or *mot*) genes with that of the donor. By this assay, it was found that SO 14 could be transformed to *mot*$^-$ at 0.3% and to *fla-6*$^-$ at 0.6%, compared to *trp-2*$^+$ at 1.2%; in all cases, the DNA solutions were used at concentrations above saturation. This finding is significant in an understanding of the fate of the competent cell after transformation.

DISCUSSION

Investigation of the genetic control of flagellation in *B. subtilis* has revealed the existence of three loci analogous to those described in *Salmonella* (8).

The ability to replace by transformation the flagellar antigen of SB 108-b with that of W 23, and vice versa, is most simply explained as the replacement of a gene specifying the flagellar antigen by its allele. Studies with *Salmonella* (7) have demonstrated that other genetic alterations can produce a similar phenotypic change in strains which undergo phase variation of their flagellar antigens. The explanation proposed above for *B. subtilis* is based upon the fact that phase variation has not been demonstrated in this species. By analogy with the terminology used with *Salmonella* (15), the structural gene for the flagellar antigen is termed *H*. Recently, it was suggested (4) that "genes determining and regulating the synthesis of flagellar antigens" be termed *hag*, in which case the structural gene would become *hagH*. As only one type of flagellin molecule is involved in the production of a given flagellum, the *H* gene of *Salmonella* has been defined (8) as the structural gene for the whole flagellin molecule and is not limited to the genetic material specifying that region of flagellin which is of antigenic importance. However, only in one case (10) has a mutation not involving the serological specificity of a flagellum been mapped within the *H* gene. *B. subtilis* SB 108-b also appears to produce only one type of flagellin molecule (R. Frankel, *unpublished data*), and a similar definition of *H* as the structural gene for the whole flagellin molecule can be tentatively adopted for this species.

Two other genes affecting flagellation were detected: a *fla* gene, controlling the presence or absence of flagella, and a *mot* gene, controlling the function of the flagella. The manner in which these genes function is unknown. The role of analogous genes in *Salmonella* in the control of flagellation has been the subject of speculation (7, 8, 11), but no suggested mode of action has been experimentally verified. It is possible to hypothesize that the three phenotypically different types of mutants described in this investigation were the result of three separate mutations within the same gene. The finding that the loci were unlinked eliminated this possibility and demonstrated the existence of three separate loci. The *fla* genes of *Salmonella* have been divided into three unlinked groups (7). In this study with *B. subtilis*, only one *fla*$^-$ mutant was examined genetically, but A. Ichiki (*personal communication*) examined many *fla*$^-$ mutants of this species and divided them into two groups, one linked to the *trp-2* locus and the other unlinked to it.

The high frequency with which transformation of the negative characteristics *mot*$^-$ and *fla*$^-$ could be obtained was of interest because of the information it provided as to the fate of the transformed cell. Nester and Stocker (17) demonstrated that the colonies resulting from the growth of individual transformed cells consisted of cells of the recipient genotype (75 to 90%), which they attributed to the transformation of only one of the two nuclei present in the competent cell at the time of DNA uptake. Such a result would predict that a colony resulting from a cell transformed with a marker conferring nonmotility would contain mainly motile progeny of the recipient type and would be scored as motile when transferred to semisolid medium. Experimentally, transformants possessing the nonmotility characteristics of the donor could be readily isolated by testing colonies grown up from single cells after DNA exposure. This indicated that either both nuclei were transformed or that the untransformed nucleus does not have a 100% chance of contributing to the progeny of the cell; as *mot*$^-$ and *fla*$^-$ transformants were isolated with a frequency of 25 to 50% of that of *trp*$^+$ transformants, the chance of contribution of the untransformed nucleus would be 50 to 75%. Recently, F. E. Young (*unpublished data*) showed that transforming DNA became attached to the forespore nucleus, suggesting that this was the nucleus which contributed to the transformed progeny. In pneumococcus, the cells present in the colony resulting from a transformed cell vary with the physiological conditions in which the cell is maintained (14). Nester and Stocker (17) induced the competent state by a method different from the one used here (2), and it is possible that the discrepancy between their results and those described was due to the different physiological conditions employed.

A method has been described (21) for the study of the replication of the genome of *B. subtilis* which can be used for the approximate mapping of loci on the total chromosome. This approach is of interest as a means to detect linkage between genes controlling flagellation and other loci and is currently under investigation.

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