Mutations affecting flagellation in *Bacillus subtilis*: their genetic analysis and use in studying the adsorption characteristics of bacteriophage PBS1.

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INTRODUCTION

Bacterial flagella provide a valuable tool in many diverse areas of research: organelle biosynthesis and regeneration, bacterial classification, immunological relationships, dynamics of motility, *in vitro* subunit reaggregation, bacteriophage-host interactions, morphology and attachment, protein thermostability, and biochemical purification. Studies have been performed with intact flagellated bacteria, with isolated detached flagella, and with purified flagellin, the protein component of the flagella.

A. Statement of Purpose

At the outset of this research (September, 1965) detailed studies of the genetic control of flagellation had only been reported in the gram-negative genus *Salmonella*. Because of the available evidence of biochemical similarity between the flagellins of various species of *Bacillus* and *Salmonella* (Weibull, 1949; Stocker, 1956), the present studies were undertaken to ascertain whether a similar genetic mechanism for the control of flagellation existed in these two taxonomically very distinct genera. *Bacillus subtilis* was chosen as a representative of the *Bacillus* genus, as it possessed a mechanism allowing genetic recombination. An attempt was made to establish the presence

and linkage relationships of loci involved in the production, the antigenic specificity, and the function of its flagella.

As a result of this study mutants of Bacillus subtilis, defective in some phase of normal motility, were isolated and provided material which made possible the investigation of the absorption specificity of the bacteriophage PBS1 (Takahashi, 1961). Various non-motile mutants, flagellated and non-flagellated, and isolated flagella preparations were examined for their ability to adsorb PBS1. The phage receptor site and several requirements for phage adsorption were defined and compared with the only previously described flagellotrophic bacteriophage, chi (Sertic and Boulgakov, 1936; Meynell, 1961), which infects sensitive Salmonella strains.

The discussion section of this thesis will include a review of relevant reports published since May, 1967, the time of completion of these studies, which deal with the genetic control of flagellation in Escherichia coli, the structure and attachment of the flagellotrophic phages (both PBS1 and chi), the susceptibility of various strains of Serratia marcescens and Escherichia coli to bacteriophage chi, and a host range mutant of phage chi able to attack Salmonella possessing the g-complex antigen.

B. Historical Context

1. Genetic control of flagellation. Flagella were

first recognized morphologically (Migula, 1897, cited in Stocker, 1956) and only later shown to be antigenically (Craigie, 1931) and biochemically (Weibull, 1948) different from the outer wall of the bacterial cell.

Flagella can be isolated by detachment from the bacterial body by shaking (Craigie, 1931; Weibull, 1948; Kobayashi, Rinker, and Koffler, 1959) or by blending in a food blender (Stocker and Campbell, 1959), and purified by differential centrifugation (Kobayashi et al., 1959; Ada, Nossal, Pye, and Abbot, 1964). Treatment with a wide variety of agents (Abram and Koffler, 1964) disaggregates the purified flagella to their monomeric form which has been shown to be proteinaceous in nature (Weibull, 1948; Astbury, Beighton, and Weibull, 1955; Ada et al., 1964.) The name "flagellin" was originally proposed (Astbury et al., 1955) as a general term for the flagellar protein but it has become limited to describe the protein molecules into which the flagella are dissociated as a result of low pH or other disaggregating procedures. Salmonella flagellin, isolated by acid dissociation of purified flagella, has a molecular weight of 40,000 (McDonough, 1965), and various preparations were considered homogeneous by the criteria of column chromatography on hydroxylapatite (Ada et al., 1964) and on DEAE-cellulose (Enomoto and Iino, 1966); cellulose acetate electrophoresis (Ada et al., 1964); disc polyacrylamide gel electro-

phoresis (Oosawa, Kasai, Hatano, and Asokura, 1966; Parish, Lang, and Ada, 1967); and ultracentrifugation (Ada et al., 1964; Kerridge, Horne, and Glauert, 1962).

Because of their importance as a public health problem, examples of the genus Salmonella have been subjected to extensive study by serological techniques. A large number of antigenically different flagella have been shown to be produced in nature by this genus (reviewed by Kauffman, 1966a). The situation was made more complex by the demonstration of the phenomenon of antigenic phase variation (Andrewes, 1922) which has been found in the majority of Salmonella serotypes. The flagella can exist as either of two alternative flagellar antigens; one is called phase-1 and the other phase-2, and an alteration between antigenic phases occurs at the measurable rate of 10^{-5} to 10^{-3}/bacterial division (Stocker, 1949). Zinder and Lederberg (1952), and subsequently Stocker, Zinder, and Lederberg (1953) and Lederberg and Edwards (1953), demonstrated that the flagellar antigen of a given serotype could be transferred to other members of the genus by transduction using phage P22, provided the serotypes in question were susceptible to this phage. However, they were only able to replace the phase-1 antigen of the recipient with the phase-1 antigen of the donor, or the phase-2 antigen of the recipient with the phase-2 antigen of the donor, and obtained no

phase-1/phase-2 interaction. From these studies they concluded that the flagellar antigen of a given phase appeared to be controlled by a single gene and that the various antigens observed were allelic forms of such a gene. They proposed the term H-1 for the gene controlling the phase-1 antigen, and the term H-2 for the gene controlling the phase-2 antigen. Subsequently, Iino (1961a) was able to demonstrate the replacement of the H-2 antigen 1,2... of a strain of Salmonella typhimurium with the phase-1 antigen b of Salmonella paratyphi B. The replacement was a very rare event and was thought to occur by unequal recombination between the H-1 locus of the donor and the H-2 locus of the recipient. The event was taken as indicative of a structural homology between the two loci.

The antigenic specificity of a flagellum is a reflection of its tertiary structure, which is determined by the primary sequence of amino acids in the component flagellin molecules. On this basis, the genes H-1 and H-2 have been redefined (Iino and Lederberg, 1964) as the structural genes for the flagellin produced in phase-1 and phase-2 respectively.

Amino acid analyses of several antigenic types of phase-1 and phase-2 flagellins have revealed the presence of N-methyl-lysine (NML), an amino acid that had not previously been found to occur in nature (Ambler and Rees, 1959).

Transductional analyses revealed that a gene which determines the presence or absence of NML is linked to but separable from the H-1 locus (Stocker, McDonough, and Ambler, 1961). Unlike H-1 and H-2, this gene determines the presence or absence of NML in both phase-1 and phase-2 flagellins and it was suggested that it coded for an enzyme which methylated some lysine radicals of already formed flagellin (Stocker et al., 1961). No such enzyme has yet been demonstrated nor has the hypothesis been definitely proven (Kerridge, 1966).

In diphasic strains, the two antigenic phases were determined at the separate loci H-1 and H-2. Therefore phase variation cannot be considered as the mutation of an antigen type determinant from one specific allele to another, but instead as the alternative manifestation of each of the two antigenic specificities already inherent in the genotype. An investigation by Lederberg and Iino (1956) into the genetic control of phase variation indicated that the H-2 gene played a decisive role in the expression of the antigenic phases. They concluded that H-2 takes two different states, active and inactive. Active H-2 was epistatic to H-1 and inhibited the production of the phase-1 antigen, while it carried out the production of the phase-2 antigen. When H-2 changed to the inactive state, corresponding to the change from phase-2 to phase-1, the production

of phase-2 antigen stopped and alternatively the production of phase-1, specified by H-1, proceeded. The expression of the H genes has been found to be regulated by several other genes: namely $\underline{ah_1}$, $\underline{ah_2}$, and $\underline{vh_2}$. $\underline{Ah_2}$ is a factor linked to H-2 which controls the activity of H-2 (Iino, 1962b): that is, the mutation of $\underline{ah_2}^+$ to allele $\underline{ah_2}^-$ causes the inactivation of H-2 and consequently the change from diphasic type to monophasic-1 type. $\underline{Ah_1}$ is a factor linked to H-1 (Iino, 1962b). The mutation to $\underline{ah_1}^-$ causes the inactivation of H-1. In such mutants H-2 activity is not affected and may therefore undergo phase variation. Consequently, in phase-1 both H-1 and H-2 are inactive and the production of flagella is entirely stopped, while in phase-2 normal flagella are produced. These mutants alternate between flagellated phase-2 and non-flagellated phase-1. $\underline{Vh_2}$ is a factor which regulates the stability of antigenic phases (Iino, 1961b) and is transduced simultaneously with H-2 at a frequency of 30%. An allele $\underline{vh_2}^-$ stabilizes H-2 in its existing state, whether inactive or active, and produces monophasic-1 or monophasic-2 types respectively. $\underline{Ah}^+$ was reported to be dominant to $\underline{ah}^-$, but the function of $\underline{ah}$ appears only in cis-position with the adjacent H (Iino, 1962b). As no other genes have been found to lie between the structural genes and their activity-controlling genes each pair behaves as two component parts of a genetic functional unit. Con-

sidering the $\underline{ah}^-$-H system analogous to the operon, the $\underline{ah}_1$ (or $\underline{ah}_2$) may correspond to the operator of H-1 (or H-2), and $\underline{ah}_1^-$ and $\underline{ah}_2^-$ to the operator negative mutants (Iino and Lederberg, 1964). This work has been pursued in greater detail by Pearce and Stocker (1967), who utilized abortive transduction to study flagellar antigens of cells heterozygous for the H-1 or H-2 regions. The partial heterozygotes produced permitted the examination of dominance and cis/trans effects, and allowed a test of the ability of a chromosomal H-2 locus to regulate the expression of H-1 and H-2 genes not located in the same chromosome. Their results indicated that H-2, or a phase-determinant locus closely linked to it, regulates expression of H-1 by determining production or non-production of an H-1-repressor substance. To explain phase variation they postulated that the activity or inactivity of the H-2 operon (H-2 structural gene and H-1-repressor locus) depended on the state of a metastable operator region, which spontaneously alternates between an 'on' or 'active' configuration, corresponding to wild-type, and an 'off' or 'inactive' configuration, corresponding to that of an operator-negative mutant.

Mutations in the H genes have been demonstrated and shown to cause changes in the flagellar antigen (Joys and Stocker, 1963) and changes in morphology (Iino, 1962a). Mutants with minor changes in the i antigen of S. typhimurium, isolated by selection in semisolid medium contain-

ing anti-*i* serum were genetically shown to be a result of mutations within the H-1 locus (Joys, 1961; Joys and Stocker, 1963). Analyses of tryptic peptide maps correlated the genetic mutation with minor changes in the flagellin molecules (McDonough, 1962). The flagella of these mutants differed antigenically from wild type but were identical to wild-type morphologically and in their ability to function in motility. Strains of bacteria have been described as possessing flagella with half the normal wavelength which functioned poorly, allowing only rotational movements in liquid medium and no translational movement in semi-solid medium. Such mutant type flagella have been termed 'curly' (Leifson and Hugh, 1953; Iino, 1962a). Curly flagella have been produced phenotypically in *Proteus mirabilis* by growth at pH 5.5 (Hoeniger, 1965) and in *S. typhimurium* by growth in the presence of para-fluoro-phenylalanine (Kerridge, 1960). Genetically stable strains with curly flagella under normal conditions of growth can be obtained, and the mutations causing the production of such flagella have been shown to be phase-specific and closely linked to the H gene of the affected phase (Iino, 1962a). The site of one mutation causing the production of curly flagella has been definitely mapped within the H-1 locus (Joys, 1961; Joys and Stocker, 1963). In support of these results Enomoto and Iino (1966) showed that the flagellin isolated from a curly mutant had one peptide different from the wild-type

flagellin when analyzed in tryptic digests. However, no serological differences have been reported between any curly mutants and their parent strains. This H mutation has therefore resulted in the production of flagella identical to the wild-type antigenically but different from the wild-type morphologically and in their ability to function in translational motility. A straight flagella mutant of S. typhimurium has also been reported (Iino and Mitani, 1966; Iino and Mitani, 1967b). The straight mutation was phase-specific in transduction studies and was therefore suggested to have originated by mutation of the structural gene of phase-2 flagellin. The flagella produced by this mutant were antigenically identical to wild-type but different from the wild-type morphologically and in their ability to function in translational motility. These mutants were non-motile in liquid and semisolid media. No biochemical examination of the flagellins of this mutant has been reported.

Both the antigenic specificity of the flagella and its morphological appearance were thus shown to be controlled by the flagellin-structural genes H-1 and H-2. The demonstration of mutations in the H gene which seemed to alter one property but not the other suggested that there were specific regions within the H gene which controlled the specific characters studied. A preliminary confirmation of the postulated presence and linear array of antigenic specificity-determining sites within the structural gene has been

obtained by intragenic recombination. Intragenic recombinants between H-2 alleles, H-2-1,2 and H-2-e,n,x (Iino, 1960 cited in Iino and Lederberg, 1964) have been obtained but the most extensive data for linear mapping of determinants has been obtained using mutant alleles of H-1-i (Joys, 1961; Joys and Stocker, 1963) and different alleles of the H-1-g complex (Yamaguchi and Iino, 1966)

The expression of the flagellin structural genes (H-1 and H-2) was also shown to be regulated by additional loci termed fla. A mutation of any fla gene from fla^+ to fla^- resulted in the loss of the ability to produce flagella in both flagellar phase-1 and phase-2. At least three groups of fla genes have been described. One group was found to be cotransducible with the H-1 locus and divisible into six cistrons on the basis of complementation tests (Joys, 1961; Joys and Stocker, 1965; Iino and Enomoto, 1966). A mutant of the second group, termed fla-29 was shown by colicin-mediated transfer to be located between gal and try (distant to H-1 or H-2) on the chromosome (Smith and Stocker, 1962). Fla-29 was shown to be identical to fla F, isolated by transduction (Iino and Enomoto, 1966). The chromosomal location of the third group, termed fla G has not been reported. Only one mutant in the fla G locus has been isolated and this differs from all other fla^- mutants studied, being the only one shown to produce flagellin but not flagella; no flagellin production could be demonstrated

in immunological studies of the other *fla*$^-$ mutants (Iino and Enomoto, 1966).

The function of the *fla* genes has been discussed (Iino and Lederberg, 1964; Joys and Stocker, 1965; Iino and Enomoto, 1966) but no mode of action has been proven with the exception of that of the *fla* G mutant. As this mutant produced a flagellin antigenically indistinguishable from the parental flagellin but was unable to form flagella, the defect seemingly affected flagellin polymerization into flagella. Different hypotheses were suggested for the other *fla*$^-$ mutants in which no antigenically measurable flagellin was produced. *Fla*$^+$ might function in the control of specific ribosomes utilized in the synthesis of flagellin, or alternatively *fla*$^+$ might produce or control the production of an internal inducer of flagellin. A specialized flagellar-forming apparatus has not yet been demonstrated, so that neither hypothesis can be proven or disproven.

Non-motile mutants have been described which possess the normal number of flagella/cell and produce flagella which do not differ from those of the wild-type motile strain in either antigenicity (Hirsch, 1947; Friewer and Leifson, 1952), morphology under the electron microscope (Enomoto, 1966a) or configuration by X-ray diffraction (Beighton, Porter, and Stocker, 1958). Both the wild-type and mutant exhibit flagellar phase variation. Such mutants have been termed 'paralyzed' (Stocker *et al.*, 1953). Mutations causing paralysis have been localized in genes

termed *mot*, shown to be genetically distinct from the flagellin structural genes, H-1 and H-2 (Enomoto, 1966a). Three complementation groups have been recognized (Enomoto, 1966a; Enomoto, 1966b) and labeled *mot* A, *mot* B and *mot* C. *Mot* C was shown to be linked to H-1 and located between *his* and H-1 on the total chromosome. *Mot* A and *mot* B are adjoining, unlinked to H-1 and located between H-1 and *leu* (*i.e.*, on the opposite side of H-1 from *mot* C. The function of the *mot* loci was discussed although it was not known whether *mot* A and *mot* B control different protein molecules or together control a single protein molecule, nor what the nature of the interaction of the three loci might be. If the bond energy of ATP was assumed to be involved in flagellar motility, the *mot* mutation did not affect the energy-generating system. The ATP content and adenosine triphosphatase activity were not significantly different in motile wild-type cells and paralyzed cells. ATP and adenosine triphosphatase activity were not detected in flagella detached from both wild-type and paralyzed cells (Enomoto, 1962, cited in Enomoto, 1966a). It was therefore suggested that the defect leading to flagellar paralysis was located in the cytoplasm, probably in the basal granules (Enomoto, 1966a); *i.e.* that the defect was in the flagellum-activating mechanisms within the bacteria rather than in the flagella themselves.

An additional flagellar mutant has been reported (termed 'slow motile' by Enomoto, 1965) which was found to have flagella quantitatively different from the wild-type. The slow motile swarmed poorly in semisolid medium but appeared as motile as the wild-type in liquid medium. Optical and electron microscopy on stained preparations of flagella as well as biochemical measurement of purified flagellar protein revealed that the mutant possessed a decreased number of flagella/cell compared with the wild-type. Genetic analyses located the mutation site of the slow motile mutant in the mot B cistron, though the mutant complemented mot B mutants. As the mutant gene affected only flagellation it was suggested to control the synthesis of flagellin polymerizing organelles from which the flagella generate (Enomoto, 1965). The slow motile mutant would therefore seem leaky, as some flagella were formed.

Evidence for the location and linkage on the Salmonella chromosome of the various loci involved in flagellation and motility has been obtained by the analyses of data from interrupted conjugation and transduction experiments. Conjugation was mediated by either F factors or colicin factors. Transduction experiments provided a means of studying both recombination and complementation through the recording of the number of swarms and trails respectively (Stocker et al., 1953; Lederberg, 1956). Table 1 lists the loci involved in the flagellation of Salmonella typhimurium, and their map

Table 1
Genetic markers of *Salmonella typhimurium*

Genetic symbol	Character controlled	Map position (min)	Reference
ah$_1$	synthesis of flagella in phase-1	55	Iino, 1962b; Iino and Lederberg, 1964
ah$_2$	synthesis of flagella in phase-2	82	
fla F	flagella presence or absence	47	Smith and Stocker, 1962; Iino and Enomoto, 1966
fla A,B,C,D,J,K	flagella presence or absence	55	Smith and Stocker, 1962; Joys and Stocker, 1965; Iino Enomoto, 1966
H-1	phase-1 flagellar antigen (flagellin structural gene)	55	Mäkelä, 1964; Iino and Lederberg, 1964; McDonough, 1965; Pearce and Stocker, 1965
H-2	phase-2 flagellar antigen (flagellin structural gene)	82	
mot A,B,C,	motility	55	Enomoto 1966a; Enomoto 1966b
nml	n-methyl-lysine in flagellar protein	55	Stocker *et al.*, 1961
vh$_2$	control rate of phase variation	82	Iino, 1961b

positions in minutes (Sanderson and Demerec, 1965). The loci which mapped at the 55 minute position were jointly transduced in the order (*mot* C, nml, ah_1) H-1--*fla* A--*fla* C--*fla* D--*fla* B--*fla* J--*fla* K (*mot* A, *mot* B). The order of loci in parentheses was not reported. H-2, vh_2, and ah_2 mapped at the 82 minute position and were jointly transduced. (Stocker *et al.*, 1961; Stocker, Smith, and Subbaiah, 1963; Joys and Stocker, 1965; Enomoto, 1966a; Enomoto, 1966b; Pearce and Stocker, 1965).

Escherichia coli has been shown to possess a single H locus (Furness, 1958), a *fla* locus (Ørskov and Ørskov, 1962; Matsumoto and Tazaki, 1967), and a *mot* locus (Armstrong and Adler, 1967). Armstrong and Adler (1967) found two groups of *mot* mutants which correspond to *mot* A and *mot* B in *Salmonella* reported by Enomoto (1966a). Crosses between *Salmonella* Hfr and *Escherichia coli* F^- demonstrated that the H-1 locus of *Salmonella* was allelic to the single H locus found in *Escherichia coli* (Mäkelä, 1964). Selection for recombinants with recombined markers on both sides of the H-2 locus demonstrated that H-2 could be incorporated with these markers. Such strains, with incorporated H-2 of the donor, showed typical phase variation, the H-2 locus being epistatic to either the H gene of the original *E. coli* parent or to the H-1 gene incorporated as a result of recombination.

The homology of *E. coli* with *Salmonella* H-1 lends support to the hypothesis of the evolution of phase varia-

tion postulated by Iino and Lederberg (1964). They suggested that the original type was a primary monophasic-1 which had H-1 but not H-2. Duplication and translocation occurred in the monophasic-1 type (such a duplication has been reported by Lederberg, 1961). The translocated H-1 was then identified as a new locus, H-2, different from the original H-1. Then, structural differentiation might have occurred between H-1 and H-2 and the strain was recognized as a diphasic strain. Subsequently, a variety of additional types evolved through mutations and deletions in the diphasic system which affected the H-1 and H-2 genes themselves, their controllers $\underline{ah_1}$ and $\underline{ah_2}$, and the variation factor $\underline{vh_2}$.

The present investigation was undertaken to establish the presence of the various loci controlling flagellation in the gram positive, transformable organism B. subtilis, and to compare these findings with genetic control in other genera, (specifically Salmonella).

2. Flagellotrophic phages. Bacteriophage chi was the first, and until very recently, the only reported flagellotrophic phage. The host range of chi was first described by Sertic and Boulgakov (1936), who reported a correlation between motility and susceptibility of Salmonella typhosa to infection by chi which lysed a motile strain but not its non-flagellated variant.

Extensive studies of chi were reported by Meynell(1961). Sensitivity to chi was assayed by the agar layer technique (Adams, 1959a) in which infection was measured by the appearance of clear plaques. Adsorption studies were also performed to establish the host range of the bacteriophage. It was shown that chi phage attacked flagellated, motile bacteria of specific antigenic type. The suggested receptor site for chi as located on the flagella was confirmed by electron microscope studies. Functional flagella of specific antigenicity were also important factors in susceptibility to adsorption and infection.

Tests with naturally-occurring strains, and with artificial serotypes to which foreign H antigens had been transduced, showed that sensitivity to chi phage depended on the H antigen (Meynell, 1961). Bacteria with the antigens of the g-complex or with antigens 1..., e,h, or Arizona 13 were resistant to the phage. Host range mutants of chi phage have been isolated which were shown to attack Salmonella having the g-group antigens (Sasaki, 1961, cited in Iino and Lederberg, 1964). The resistance of the g-complex was shown to be phase specific (Meynell, 1961). When g.. antigen was introduced into diphasic strains by transduction of H-1-g.., the resulting transductional clones were resistant to chi phage in phase-1 while sensitive in phase-2. The antigenic phase specificity of the resistance was also observed on a motile chi-resistant mutant of S. typhimurium (Sasaki, 1961,

cited in Iino and Lederberg, 1964). The mutant was shown to be resistant in phase-2, 1,2-type, while sensitive in phase-1, i type. The determinant of the phage resistance and the 1,2-type determinant, H-2, were always co-transduced.

Tests with non-motile flagellated (i.e., paralyzed) as well as non-flagellated bacteria showed these strains to be resistant to chi phage (Meynell, 1961). Adsorption was diminished by removal of flagella from a sensitive strain or by artificially paralyzing the strain by treatment of the bacteria with chloral hydrate, 2,4-dinitrophenol, aureomycin, formaldehyde, or heat at 56 C for 30 min (Meynell, 1961). No phage were found to be adsorbed by detached flagella (Meynell, 1961).

Plaques of chi phage on the slow motile mutant of Salmonella typhimurium were small and cloudy, whereas on the wild-type they were large and clear. The efficiency of plating on the mutant was 0.36 as compared with 1 for the wild-type (Enomoto, 1965). Meynell (1961) reported that a curly mutant of S. abortus-equi showed a sensitivity to chi phage on a spot plaque assay test but failed to adsorb the phage. No detachable loss of phage from the supernatant occurred following incubation of chi and the curly mutant. Preliminary reports of a straight flagellar mutant indicated that it was sensitive to bacteriophage-chi (Iino and Mitani, 1966a).

Adsorption of the transducing bacteriophage PBS1 by Bacillus subtilis was reported (Joys, 1965) to show a correlation with motility as a non-motile strain of Bacillus subtilis was shown to be insensitive to PBS1. On this basis it was suggested that PBS1 was a second example of a flagellotrophic phage.

The present investigation further elucidated the adsorption specificity of phage PBS1 by quantitatively measuring its adsorption to non-motile B. subtilis strains, various flagellar mutants, and isolated detached flagella.

Partial reports of these investigations into the adsorption specificity of phage PBS1 and into the genetic control of flagellation have been published (Frankel and Joys, 1966; Joys and Frankel, 1967).

MATERIALS AND METHODS

Materials:

A. Strains
1. *Bacillus subtilis*

W 23	W 23 wild-type (Burkholder and Giles, 1947)	The culture was obtained from J. Spizizen (Scripps Clinic, LaJolla, Calif.)
SB 108	*fla*$^-$ derivative of strain 168	The culture was obtained from T.M. Joys stock of SB 108 obtained from E.W. Nester (Univ. of Washington, Seattle)
SB 108-b	*fla*$^+$ mutant of SB 108	The culture was obtained from T.M. Joys who had isolated the strain as a spontaneous motile mutant of SB 108. The strain was obtained from a single clone in semisolid medium.
MS 10	*his*$^-$ *str*^{r} derivative of 168	The culture was obtained from T.M. Joys stock of MS 10 obtained from the collection of J. Spizizen.
SO 4	*fla*$^-$ mutant of SB 108-b	Stable non-flagellate mutant obtained following ultraviolet irradiation
SO 5	paralyzed mutant of SB 108-b	Non-motile mutant in both liquid and semisolid medium, yet shown to be flagellated by Leifson's staining method and to be agglutinated with rabbit anti-flagellar serum prepared against SB 108-b. The mutant was obtained following ultraviolet irradiation.

SO 6 *fla*$^-$ mutant of SB 108-b	Stable non-flagellate mutant obtained following ultraviolet irradiation
SO 7 *fla*$^-$ mutant of SB 108-b	Stable non-flagellate mutant obtained following ultraviolet irradiation
SO 9 *fla*$^-$ mutant of SB 108-b	Stable non-flagellate mutant obtained following ultraviolet irradiation
SO 12 flagellar antigen transformant of SB 108-b	DNA from strain W 23 was used to transform strain SB 108-b. Serum-semisolid medium containing anti-SB 108-b serum, was used as the selection method.
SO 14 *str*^{r} transformant of SB 108-b	MS 10 DNA was used to transform strain SB 108-b to *str*^{r}.
SO 49 paralyzed transformant of SO 14	SO 5 DNA was used to transform strain SO 14 to paralysis
SO 55 paralyzed transformant of SO 12	SO 5 DNA was used to transform strain SO 12 to paralysis.
SO 65 *fla*$^-$ mutant of W 23	Stable non-flagellate mutant obtained following ultraviolet irradiation.
SO 67 flagellar antigen transformant of W 23	SB 108-b DNA was used to transform strain W 23. The flagellar antigen of the donor was selected by serum-semisolid medium containing anti-W 23 serum.

The genetic descriptions and relevant phenotypes of the *Bacillus subtilis* strains are summarized in Table 2 using the nomenclature of Demerec, Adelberg, Clark, and Hartman, 1966.

Table 2

Strains of *Bacillus subtilis*

Strain	Derivation	Mutant Loci and Mutation Sites											Relevant Phenotype*					
		trp -2	his -2	H	fla -1	fla -4	fla -6	fla -7	fla -9	fla -48	mot	str	Trp	His	Antig	Flag	Paral	Str
W 23	W 23 wild type	+	+	H-b	+	+	+	+	+	+	+	+	+	+	b	+	-	s
SB 108	*fla*⁻ derivative of 168	-	+	H-a	-	+	+	+	+	+	+	+	-	+	(a)	-	(-)	s
SB 108-b	*fla*⁺ derivative of SB 108	-	+	H-a	+	+	+	+	+	+	+	+	-	+	a	+	-	s
MS 10	*his*⁻*str*ʳ derivative of 168	-	-	H-a	+	+	+	+	+	+	+	-	-	-	a	+	-	r
SO 4	*fla*⁻ mutant of SB 108-b	-	+	H-a	+	-	+	+	+	+	+	+	-	+	(a)	-	(-)	s
SO 5	paralyzed mutant of SB 108-b	-	+	H-a	+	+	+	+	+	+	-	+	-	+	a	+	+	s
SO 6	*fla*⁻ mutant of SB 108-b	-	+	H-a	+	+	-	+	+	+	+	+	-	+	(a)	-	(-)	s

Table 2 (con't)

SO 7	fla⁻ mutant of SB 108-b	-	÷	H-a	÷	+	+	-	+	+	+	+	-	+	(a)	-	(-)	s
SO 9	fla⁻ mutant of Sb 108-b	-	+	H-a	+	+	+	+	-	+	+	+	-	+	(a)	-	(-)	s
SO 12	W 23 -X SB 108-b**	-	+	H-b	+	+	+	+	+	+	+	+	-	+	b	+	-	s
SO 14	MS 10 -X SB 108-b	-	+	H-a	+	+	+	+	+	+	+	-	-	+	a	+	-	r
SO 49	SO 5 -X SO 14	-	+	H-a	+	+	+	+	+	+	-	-	-	+	a	+	+	r
SO 55	SO 5 -X SO 12	-	+	H-b	+	+	+	+	+	+	-	+	-	+	b	+	+	s
SO 65	fla⁻ mutant of W 23	+	+	H-b	+	+	+	+	+	-	+	+	+	+	(b)	-	(-)	s
SO 67	SB 108-b -X W 23	+	+	H-a	+	+	+	+	+	+	+	+	+	+	a	+	-	s

*Trp, tryptophan; His, histidine; Antig, flagellar antigen; Flag, flagellated; Paral, paralyzed; Str, streptomycin; r, resistant; s, susceptible. Arbitrary numbers starting from 1 were used to label the fla loci as isolated; they do not indicate genetic non-identity.

**The symbol W 23 -X SB 108-b indicates that DNA from strain W 23 was used to transform strain SB 108-b (Nester, Schafer, and Lederberg, 1963).

2. Phage. Phage PBS1-V1 was used. This strain was isolated by T.M. Joys as a virulent mutant of phage PBS1 obtained from C. Anagnostopoulos (Laboratoire de Genetique, Gif-sur-Yvette, Seine et Oise, France).

B. Stock Solutions

Glassware was washed in 7 X detergent (Linbro Chemicals) containing EDTA to minimize copper contamination which has a detrimental effect on transformation (J. Spizizen, personal communication). Glass-distilled and deionized water was used for media and reagent preparation and for all glassware rinsing procedures. The chemicals used, unless indicated otherwise, were Baker or Merck analyzed reagent grade. Solutions and media were stored at 4 C.

1. 10 X Minimal Salts

distilled water	1 liter
ammonium sulfate	20 g
dipotassium phosphate	140 g
monopotassium phosphate	60 g
sodium citrate · $2H_2O$	10 g
magnesium sulfate · $7H_2O$	2.0 g

The salts were added, with constant mechanical stirring, to 1 liter of distilled water in a flask. Once the solution was clear it was poured into pyrex bottles and autoclaved at 250 F for 20 min.

2. 50% Glucose

dextrose	50 g
distilled water to	100 ml

The components were mechanically mixed until the glucose was completely dissolved. The solution was sterilized by passage through a 0.45 μ Millipore filter.

3. 2 mg/ml L-tryptophan

L-tryptophan	0.2 g
distilled water	100 ml

The L-tryptophan (Calbiochem) was added to the distilled water, mixed mechanically and sterilized by passage through a 0.45 μ Millipore filter.

4. Magnesium sulfate 0.5 M

magnesium sulfate	12.3 g
distilled water	to 100 ml

Distilled water was added to magnesium sulfate, the solution mixed mechanically and sterilized by passage through a 0.45 μ Millipore filter.

5. Casein hydrolysate

a. 5% for adding to transformation plates

casein hydrolysate	5 g
distilled water	to 100 ml

Distilled water was added to casein hydrolysate, the solution was mixed mechanically and sterilized by autoclaving. The casein hydrolysate used was vitamin-free and salt-free, as supplied by Nutritional Biochemicals, Cleveland, Ohio, for investigational use.

b. 5% for transformation media used in the preparation of competent cells.

10% sterile solution of vitamin-free casein hydrolysate supplied by Nutritional Biochemicals for microbiological procedures, was diluted with sterile distilled water to make a 5% solution.

6. Sodium Chloride 2 M

sodium chloride	11.7 g
distilled water	to 100 ml

Distilled water was added to the sodium chloride, the solution mixed mechanically and sterilized by autoclaving.

7. Sodium Citrate 1 M

sodium citrate	29.4 g
distilled water	to 100 ml

Distilled water was added to the sodium citrate, the solution mixed mechanically and sterilized by autoclaving.

8. Leifson's staining stocks
   a. 1.2% Basic fuchsin (Allied Chemical, special grade for flagella staining) in 95% Ethyl alcohol
   b. 3.0% Tannic acid in distilled water
   c. 1.5% NaCl in distilled water

C. Working solutions

L-Tryptophan	Stock (2 mg/ml)	distilled water
a. 1000 µg/ml	20 ml	20 ml
b. 500 µg/ml	10 ml	30 ml
c. 100 µg/ml	2 ml	38 ml
d. 50 µg/ml	1 ml	39 ml

2. Magnesium sulfate 0.05 M
   The stock 0.5 M magnesium sulfate solution was diluted 10 fold with sterile distilled water.

Casein hydrolysate	Stock*	distilled water
a. 0.2%	2 ml	48 ml
b. 0.1%	1 ml	49 ml

*casein hydrolysate 5% stock preparation of competent cells (solution 5b)

D. Media
1. Liquid
   a. 1 X minimal - glucose

10 X minimal salts	10 ml
distilled water (sterile)	90 ml
50% glucose	1 ml

The components were sterilized separately, 10 X minimal and water by autoclaving, 50% glucose by Millipore filtration (0.45 µ filter), and mixed aseptically.

b. Four-hour medium

Working stock solution	volume added for 10 ml	final concentration
L-tryptophan 50 µg/ml	1 ml	50 µg/ml
casein hydrolysate 0.2%	1 ml	0.02%
magnesium sulfate 0.05 M	1 ml	0.005 M
1 X minimal-glucose	7 ml	

The medium was made fresh for each day's experiments.

c. 90 minute medium

Working stock Solution	volume added for 10 ml	final concentration
L-tryptophan 50 µg/ml	1 ml	5 µg/ml
casein hydrolysate 0.1%	1 ml	0.01%
magnesium sulfate 0.05 M	1 ml	0.05 M
1 X-minimal-glucose	7 ml	

The medium was mixed fresh daily.

If the organism to be grown was his⁻ and try⁻ 0.5 ml 1000 µg/ml tryptophan and 0.5 ml 1000 µg/ml histidine was used in the four-hour medium. The 90-minute medium was made up with 0.5 ml. 100 µg/ml tryptophan and 0.5 ml 100 µg/ml histidine.

d. Medium d for DNA preparation for 1500 ml

distilled water	1350 ml
10X minimal salts	150 ml
5% casein hydrolysate	15 ml
L-tryptophan	15 ml
50% glucose	15 ml

The distilled water was autoclaved in a 2800 ml flask, allowed to cool and the remaining components added. To minimize the chance of contamination, the four Millipore-filtered components were combined and added as one solution to the flask.

e. Sucrose-saline citrate-lysozyme
for 50 ml

sucrose 20%	40 ml
2 M sodium chloride	2.5 ml
1 M sodium citrate	2.5 ml
lysozyme (Sigma grade 3X crystalized)	5 mg (final concentration 100 µg/ml)

f. Saline - citrate

2 M NaCl	5 ml
1 M Na citrate	5 ml
distilled water	90 ml

g. Penassay broth

Penassay medium (Difco)	1.75 g
distilled water	100 mg

Prior to storage, the solution was incubated over night at 37 C and examined visually for sterility. The component were mixed and sterilized by autoclaving.

2. Non-liquid
a. minimal medium plates
solution - 1

agar	4 g
distilled water	1180 ml

The agar was added to the distilled water in a 500 ml Erlenmeyer flask and sterilized by autoclaving for 20 min at 250 F. The solution was cooled to 55 C in a water bath.

solution - 2

10X minimal salts	20 ml
50% glucose	2 ml

The sterilized components of solution 2 were placed in a 55 C water bath. Solution 1 and solution 2, both at 55 C., were combined aseptically and mixed well. Approximately seven 100 mm sterile plastic petri dishes were prepared from each 200 ml of solution. The plates were allowed to solidify at room temperature and were then inverted

and incubated overnight at 37 C prior to use. Extra plates, once incubated, were stored for several days at 4 C.

Auxotrophs were grown on plates containing minimal salts medium and the required amino acid(s). 0.1 ml of a stock 2 mg/ml amino acid solution was spread with a glass spreader on each preincubated minimal salts plate. The plates were allowed to dry before use.

b. Tryptose blood agar (TBAB) plates

tryptose blood agar base (Difco)	3.3 g
distilled water	100 ml

The dehydrated medium was added to the distilled water, mixed well and autoclaved at 250 F for 20 min. After cooling to 55 C in a water bath the solution was poured into seven 100 mm sterile plastic petri dishes. The plates were allowed to solidify at room temperature and were then inverted and incubated overnight at 37 C. Following incubation, plates were stored at 4 C until needed.

c. semisolid medium

1. for phage overlay

Penassay medium (Difco)	1.75 g
agar	0.6 g
distilled water	100 ml

The components were mixed and sterilized by autoclaving. The solution was cooled to 55 C in a water bath and was well shaken prior to use. Fresh medium was made for each day's experiments.

2. for motility studies

Penassay medium	1.75 g
agar	0.3 g
distilled water	100 ml

The components were mixed and sterilized by autoclaving (250 F, 20 min). The sterile solution was cooled to 55 C in a water bath and then approximately 10 ml of solution was poured into each 60 mm sterile plastic petri dish or 1 ml volumes were pipetted into the cups of hemagglutination trays sterilized by ultraviolet irradiation. The medium was allowed to solidify at room temperature and the dishes were then incubated overnight in an upright position at 37 C. The semisolid medium was made up fresh for each set of plates. Poured petri dishes but not hemagglutination trays were stored, once incubated overnight, for several days at 4 C.

E. Leifson's flagella stain

The stain solution was prepared by mixing equal parts of the three stock solutions. The stain solution was ready for use immediately and was stored for several months at 4 C. The stain solution was discarded when staining time exceeded that of freshly prepared stain by more than 5 min.

Methods:

A. Phage

1. Preparation of phage lysate. Lysates of phage PBS-V1 were made following the method of Takahashi (1963). Phage-sensitive cultures of strain SB 108-b were grown in Penassay Broth (Difco) with rapid shaking at 37 C until logarithmic growth phase was reached as determined by opti-

cal density measurements in a Klett-Summerson colorimeter fitted with a red filter. To determine viable count the Klett value was compared with a standard growth curve in which Klett values were plotted against viable count. The culture was then infected with phage at a multiplicity of 1.0. After incubation at 37 C on a shaker for one hour the infected culture was incubated, without shaking overnight. Following centrifugation at 3,000 g for 5 min to deposit cells, the supernatant (=lysate) was filtered through a Millipore filter (0.45 μ membrane pore size).

2. Plaque assay. A modification of the agar-overlay technique (Adams, 1959a) was used to titrate phage lysates and to measure the number of phage in the supernatant following adsorption (Adams, 1959b). TBAB plates kept at room temperature were inoculated with 0.1 ml of lysate dilution (or supernatant dilution following adsorption), 10. ml SB 108-b (log phase culture of indicator strain), and 3.0 ml of semisolid Penassay Broth autoclaved and cooled to 55 C. The plate was swirled to mix the components, allowed to solidify at room temperature, and then incubated in an upright position overnight at 37 C. Penassay Broth alone, without the phage lysate, was used as a control.

3. Phage adsorption. Penassay Broth cultures of the strains to be tested were grown with shaking at 37 C to logarithmic phase. The cultures were examined by phase-contrast microscopy for the presence or absence of motility. Equal volumes of each culture were mixed with

phage (multiplicity of infection approximately 1.0), and the mixture shaken at 37 C for 20 min. The control consisted of a mixture of equal volumes of Penassay Broth and phage lysate. The cells (and with them any adsorbed phage) were centrifuged at 3,000 g for approximately 2 min and the supernatant fluid collected. Appropriate dilutions of the supernatant fluid were made in Penassay Broth and assayed for phage by the agar-overlay technique. The percent adsorption was determined by a comparison of the supernatant and control titers.

For cell-free flagella preparations, equal volumes of the required phage dilutions (to give 4 x 10^6 infective units/ml and the flagella suspension to be tested were mixed and incubated at 37 C with shaking for 20 min; the mixture was then titrated by the agar-overlay technique for infectious particles.

B. Isolation of non-motile mutants.

A procedure of ultraviolet irradiation for the isolation of sporulation mutants (J. Spizizen, personal communication) was used. A standard growth curve was made by inoculating SB 108-b from an overnight TBAB plate into a Nephelo culture flask (Bellco Glass Company) containing Penassay Broth (Difco). The flask was shaken in a 37 C water bath. Optical density was read at hourly intervals in a Klett-Summerson colorimeter fitted with a red filter, and samples were taken for viable counting.

An ultraviolet irradiation kill curve was determined for cultures in log phase (optical density of 140 Klett units corresponding to 4 x 10^8 viable cells/ml). 10 ml of the log phase broth culture was placed in an open 100 mm plastic petri dish 12 inches under a Sylvania germicidal ultraviolet lamp (Thomas Co. bulb type A catalog number 63220E). At specified times 0.5 ml samples were removed, diluted in Penassay Broth, and 0.1 ml of the appropriate dilution was spread on a TBAB plate. Plates were inverted and incubated overnight at 37 C. Colony counts were then performed and a graph of surviving viable cells versus time of irradiation was plotted.

For the isolation of non-motile mutants, a log phase Penassay Broth culture of SB 108-b containing approximate 4 x 10^8 cells/ml was irradiated under conditions producing 99.9% loss of viable cells in the test experiment. A sample was removed and diluted 1:20 into Penassay Broth contained in a flask. The flask was wrapped in aluminum foil to minimize photoreactivation by visible light. The flask was shaken overnight in a 37 C water bath. The overnight culture was irradiated under the same conditions of ultraviolet exposure as had been used for the initial log-phase culture, and again a sample was removed, diluted 1:20, and incubated in a foil-wrapped flask overnight. This third overnight culture was similarly treated (irradiation, dilution of sample, overnight incubation).

The final overnight culture was diluted in Penassay Broth and plated for single colonies on TBAB plates. After overnight incubation at 37 C the colonies were individually picked, with sterile toothpicks, to semisolid medium (0.3% agar) contained in the cups of sterile plastic hemagglutination trays. Colonies showing no evidence of movement through the medium after overnight incubation were tested for the stability of their non-motile character by repeated incubations in semisolid medium contained in 60 mm plastic petri dishes. The presence or absence of flagella was tested by agglutination with specific rabbit antiflagellar serum and by direct staining.

C. Isolation of flagella.

Flagella were detached by the blending technique of Stocker and Campbell (1959) and purified according to the method of Kobayashi et al. (1959) and Ada et al. (1964).

To obtain a maximally flagellated culture, SB 108-b was passed three times through semisolid medium, the leading edge of the swarming growth used to inoculate each subsequent plate. A Penassay Broth suspension of the highly motile culture was used to inoculate 500 ml volumes of TBAB contained in enamel trays 30 cm by 20 cm by 5 cm. The trays were incubated at 37 C for 40-45 hr. Growth was harvested into 20 ml of 0.9% NaCl and the suspension blended in a Waring Blendor for 90 sec. Cells were deposi-

ted by centrifugation at 6,000 g for 30 min. The supernatant fluid, called the "blender supernatant fluid," contained 4.4 mg/ml of protein when assayed by the Lowry test (Lowry, Rosebrough, Farr, and Randall, 1951), with Bovine Serum Albumin (Calbiochem grade A) as standard.

Samples of this supernatant fluid were further centrifuged at 40,000 g for two hours to deposit the flagella and the pellet was resuspended in distilled water. This suspension showed concentration to 6.8 mg/ml of protein by the same test, and was found to contain typical flagella-like filaments when examined with the electron microscope. Unstained and uranyl acetate stained preparations appeared to have identical morphology.

D. Serological Methods.

1. H antigen suspension. A method for preparing H antigen suspensions similar to that reported for Salmonella (Kauffman, 1966b) was used. A log-phase Penassay Broth culture was prepared containing approximately 4 x 10^8 cells /ml determined by comparison with the Klett vs. viable count plot, and its motility examined by phase-contrast microscopy. An equal volume of formalinized saline (0.6% formalin in 0.9% saline solution) was added to the motile culture. Following overnight incubation at 37 C the suspension was tested for its sterility by overnight

incubation at 37 C of a 1.0 ml sample spread on a TBAB plate or a sample of the suspension inoculated into Penassay Broth. The sterile H antigen suspension was stored at 4 C.

2. Preparation of anti-flagellar serum. A method similar to that reported for Salmonella by Kauffman (1966c) was used to prepare specific anti-flagellar serum in rabbits. Rabbits were immunized with four intravenous injections of H suspension into the outer marginal ear vein according to the following schedule:

Day 1	0.5 ml
5-7	1.0 ml
10-12	2.0 ml
15-17	3.0 ml

Blood was collected from the ear 7 to 10 days after final immunization. Second and third blood samples were collected at weekly intervals as necessary. The blood was collected in glass test tubes and allowed to clot at room temperature. The clot was ringed and the tube incubated overnight at 37 C to allow for clot contraction. Serum was obtained following centrifugation at 20,000 g for 15 minutes. The serum was filtered by passage through a Millipore filter (0.45 μ pore size) prewetted with sterile 0.9% saline. The serum was titrated by the slide micro-agglutination method (see below) and stored at -20 C.

3. Serum titration - slide microagglutination method. A slide microagglutination method suggested by T.M. Joys (later published as Joys and Stocker, 1966) was used for the tit-

ration of anti-flagellar serum. The serum to be titrated was diluted in 0.9% saline, doubling dilutions generally used. H antigen agglutinable suspension was made by adding 40% formalin to a final concentration of 0.3% to a log-phase broth grown culture (approximately 4 x 10^8 cells/ml). The agglutinable suspensions were stored at 4 C and shaken before use in titrations. Standard loopfuls (approximately 0.02 ml) of the serum dilution and the H antigen agglutinable suspension were mixed on an acid-washed, alcohol-cleaned glass slide. The slide was placed on a glass support in a large glass petri dish in the bottom of which had been placed a moistened piece of filter paper. The dish was covered and incubated at 37 C for two hr and then at room temperature for one hr. Agglutination was observed macroscopically and under a dark field microscope.

4. Serum absorption. The method of Kauffman (1966d) was used for the preparation of absorbing suspensions and the absorption of antiserum. An absorbing suspension was made by inoculating a highly motile colony into Penassay Broth. After overnight incubation at 37 C, the culture obtained was used as the inoculum for a 30 cm by 20 cm by 5 cm enamel tray containing approximately 500 ml of TBAB medium. The tray was incubated for 40-48 hr and its growth harvested into a minimal amount of sterile 0.9% NaCl. This suspension was termed an absorbing suspension. No further

purification was attempted as Stocker and Campbell (1959) had demonstrated that the harvesting procedure detached many flagella.

The antiserum to be absorbed was mixed at 1:5 dilution with the appropriate absorbing suspension and incubated without shaking 2 hr in a 37 C water bath. Following incubation the cells were removed by centrifugation at 20,000 g for 20 min. The supernatant solution (i.e, the absorbed serum) was tested for its residual agglutinating ability by the slide microagglutination method using the appropriate H agglutinable suspensions as antigens. If additional absorptions were necessary to remove a particular agglutinating ability completely, the above procedure was repeated utilizing 1:1 dilutions of antiserum to absorbing suspension.

E. Direct staining of flagella. The technique described by Leifson (1960) was followed.

1. Bacterial suspension. Cells were grown in Penassay Broth or resuspended from a solid surface into distilled water. Similar staining results were obtained whether or not formalin, to a final concentration of 5-10%, was added to the bacterial suspension as suggested by Leifson (1960). The specimen was centrifuged at 3,000 g for 3-5 min to sediment the cells. The pellet was washed twice with distilled water by repeated centrifugation. The final

pellet was resuspended in distilled water to obtain a light suspension.

2. Slides. Clean grease-free slides were found to be essential and were obtained by cleaning the slides in sulfuric acid-potassium dichromate cleaning solution. The slides were then thoroughly washed, first in tap water and then in distilled water to remove all traces of acid. The slides were air-dried and stored in dust-free containers. Satisfactory stains were also obtained by using slides which were pre-cleaned by the manufacturer (Gold Seal slides, Clay Adams Inc., New York). Immediately before use, the slides were heated in a Bunsen burner and then allowed to cool.

A loopful of the prepared suspension was placed at one end of the slide; the slide was then tilted, and the fluid allowed to flow to the opposite side. Two smears were made side by side by inoculating on either side of a line drawn with a wax pencil across the middle of the slide, parallel to the long edge. The smears were allowed to air-dry before being stained.

3. Staining. Stain solution was applied to the slide with a Pasteur pipette. Each smear was flooded separately, the stain not being allowed to spread beyond the wax line nor to run off the slide. The staining usually took 5 to 15 min and was judged complete when a colloidal precipitate formed on the surface of the slide. When the

staining time had elapsed the slide was placed directly under a gentle stream of water. After washing, the slide was allowed to drain dry or was carefully blotted. When viewed microscopically, flagella stained in this manner appeared red.

F. Transformation

A modification of the method of Anagnostopoulos and Spizizen (1961) was 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 (1963) method was used.

1. Preparation of DNA solution. A TBAB plate was heavily streaked with an inoculum obtained from the confluent overnight growth of the organism on a TBAB plate. The streaked plate was inverted and incubated at 37 C for 5 to 7 hr. The growth was then harvested into 5 ml of medium d and this suspension was used to inoculate 1,500 ml of medium d in a 2,800 ml flask. Following overnight incubation on a 37 C shaker the culture was harvested by centrifugation at 5,000 g for 15 min at 4 C. The packed cells were resuspended with a tissue homogenizer, into 25 ml (1:60 concentration) of sucrose-saline citrate-lysozyme solution (solution e). The suspension was placed in a 37 C waterbath and incubated, without shaking, until

phase-contrast microscopic examination revealed that approximately 90% of the cells had been protoplasted. The protoplasts were harvested by centrifugation at 20,000 g for 15 min at 4 C. The green mucoid pellet obtained was resuspended into 25 ml saline citrate (solution f) using a tissue homogenizer. The suspension was brought to 37 C in a water bath and the following stepwise additions and incubations performed:

Solution	Volume Added	Incubation at 37 C
ribonuclease*	1.25 ml	25-30 min
trypsin**	1.25 ml	30 min
4 M NaCl, 10% desoxycholate	25 ml, 0.5 ml	30-60 min

*1 mg/ml solution freshly made in distilled water; Sigma type 1-A, 5 X crystallized.

**1 mg/ml solution; Sigma type 1, 2 X crystallized.

The suspension was then stored at 4 C overnight. Debris and degradation products were removed by centrifugation at 20,000 g for 15 min at 4 C. The supernatant was collected and alcohol precipated by the slow addition of the supernatant to 10X volume of cold 95% ethyl alcohol, performed in an ice bath with the solution constantly stirred. The DNA fibers were collected onto a glass rod, blotted on filter paper to remove excess alcohol, and suspended into 25 ml 2 M NaCl solution. The suspension was passed through a pre-wetted Millipore filter (0.45 μ pore size) fitted with a pre-filter pad. The filtered suspension was stored at 4 C and shaken daily. After one week at 4 C the supernatant of the suspension

was used as the DNA preparation in transformation. When stored at 4 C the DNA preparation retained its original transforming activity for many months.

2. Preparation of competent cells. A loopful of confluent overnight growth was inoculated into 2.5 ml of four-hour medium, mixed well and shaken for 4 hr at 37 C. The culture was spun at 3,000 g for 5 min and the resultant pellet resuspended in the original volume of 1 X minimal-glucose. This suspension was then diluted 1:10 into 90-minute medium and shaken for 90 min at 37 C.

3. Transformation of biochemical markers. One-tenth volume of DNA solution was added to the competent cell culture and the suspension shaken at 37 C for 30 min. Dilutions were made in 1 X minimal-glucose solution and 0.1 ml of each appropriate dilution plated onto minimal medium plates. Viable counts were made on the 10^{-5} dilution plated onto minimal medium supplemented with the amino acids(s) required for growth by the recipient strain. Plates were inverted and incubated at 37 C for two nights. For tryptophan transformation experiments the addition of 0.1 ml of 5% casein hydrolysate to each plate permitted the reading of the plates after overnight incubation at 37 C.

The percentage of transformation was determined by comparing the colony counts on the 10^{-2}, 10^{-3} and 10^{-5} plates as follows:

$$\text{Percent of Transformation} = \frac{\text{number of transformants*}}{\text{viable count**}} \times 100$$

*transformants = colonies counted on 10^{-2} or 10^{-3} unsupplemented plate.

**viable count = colonies counted on 10^{-5} supplemented plate.

4. Motility and H antigen transformation. Competent cultures were exposed to one-tenth volume of DNA solution for 30 min at 37 C, with shaking. The suspension was then suitably diluted and 10 standard loopfuls (approximately 0.02 ml/loop) of each dilution were individually inoculated into semisolid medium (0.3% agar) contained in 1 ml volumes in wells of sterile plastic hemagglutination trays. At dilutions at which about half of the inoculated loopfuls gave rise to swarms, each swarm was considered to have arisen from one original motile transformant (Stocker et al., 1953). The data obtained from these dilutions were used to calculate the number of cells transformed to motility.

To select for transformants with the specific flagellar antigen of the donor, 0.1 ml sterile rabbit anti-flagellar (anti-H antigen) serum prepared against the recipient was added to the semisolid medium. The antiserum was diluted in the procedure to prevent interactions with antibodies directed against the somatic antigens.

5. Linkage measurement. Linkage tests were made by the method of Nester (Nester and Lederberg, 1961; Nester, et al., 1963). Experiments in which the linkage of markers

was to be studied were performed in triplicate utilizing different DNA concentration in the transformation mixture. 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 (r).

The co-transfer index (r) is a measure of the frequency of joint transfer of two markers compared to the total number of recombinant genotypes measured in the transformation experiment.

$$r = \frac{a'b'}{a'b' + a'b^{\circ} + a^{\circ}b'}$$

$$= \frac{a'b'}{a' + b' - a'b'} \quad \begin{matrix}(\text{since } a' = a'b^{\circ} + a'b') \\ (\text{and } b' = a^{\circ}b' + a'b')\end{matrix}$$

In a transformation involving the transfer of DNA from an a'b' donor to an a°b° recipient, the transformants in the experiment are symbolized as a'b', a'b° a°b'. If the value obtained for r did not exceed 0.10 in repeated experiments and utilizing DNA at different concentrations, the markers under study were considered to be unlinked.

RESULTS

A. Genetic control of flagellation in Bacillus subtilis.

Serological analysis of Bacillus subtilis

Historically, serological analysis of bacteria has been performed by workers in medically related fields as a means of subdividing the genera and species of the pathogenic bacteria. Following biochemical and cultural characterization into groups (genera and species) the strains are analyzed serologically, usually by the examination of residual agglutinating properties of absorbed antiserum (reviewed by Kauffmann, 1966a). Bacteria not considered to be of prime medical importance have been classified by bacterial taxonomists on the basis of biochemical and morphological analyses and have generally not been further divided into serological subgroups. Consequently, there were no published reports of the antigenic properties of the organism used in this study, Bacillus subtilis, at the time this research was initiated.

As genetic experiments involving bacterial flagella depend to a large extent on the use of anti-flagellar sera for the assay of flagella and the selection of one flagellar antigen type over another, an analysis of the antigenic properties of B. subtilis was a prerequisite

for the genetic studies. As no guidelines for the use of this organism in serological tests were available, the well defined methods for preparing H-agglutinable suspensions and antiserum and for titrating and absorbing the antiserum in the genus Salmonella (Kauffmann, 1966b; Kauffmann, 1966d) were utilized and found to work well.

For study of the structural gene for Bacillus subtilis, termed H by analogy with Salmonella, allelic forms of the gene, specifying different flagellar antigens were necessary. Although such forms could probably be obtained by serum/ semisolid selection for mutants of a wild-type H antigen (as shown in Salmonella by Joys and Stocker, 1963) such differences are usually minor and often difficult to work with. Consequently, as a preliminary screening technique, two transformable strains of B. subtilis, SB 108-b (a derivative of the well characterized strain 168) and W 23 were chosen. They were expected to show antigenic difference because the known physiological differences between the two strains indicated that they were not closely related.

Antisera prepared in rabbits against formalinized broth cultures of B. subtilis strains SB 108-b and W 23 were titrated on the strains used in immunization and on non-motile (flagellaless) derivatives of such strains. High titers of agglutination, analogous to the Salmonella

system, were obtained on reaction of each antiserum with the motile strain used in its preparation. Agglutination was obtained, but at a lower titer, for the reaction between antiserum and its homologous non-flagellated suspension, indicating that the formalinized broth cultures used in immunization had elicited anti-somatic as well as anti-flagellar antibodies. The existence of both somatic and flagellar antigens was confirmed for each strain by absorption of the antiserum with the homologous non-flagellated strain. The results are summarized in Table 3. It can be seen that each anti-flagellar serum when absorbed with the homologous non-flagellated strain was unaffected in its titer on a homologous flagellated suspension (allowing for dilution in absorption) while the activity against a homologous non-flagellated suspension was completely removed. Each antiserum was thus shown to possess both anti-somatic and anti-flagellar antibodies.

The titers of each serum against the heterologous strains, flagellated and non-flagellated, are also shown in Table 3. Anti-W 23 serum was found to agglutinate both flagellated and non-flagellated strains of SB 108-b. Similarly, anti-SB 108-b serum agglutinated both flagellated and non-flagellated W 23 *fla*$^{+}$ strains. For both sera, the agglutination titer was lower on the hetero-

Table 3

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

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

Table 3 (con't)

SO 65	1:6	12	10	10	3	-	10	12
SB 108	1:24	12	-	-	3	-	3	12
SO 12	1:12	12	-	-	-	-	-	12
SO 67	1:12	10	10	10	-	-	10	-
SO 67 + SB 108	1:24	-	-	-	-	-	-	-

*Antisera were tested by the micromethod before and after complete absorption. Their titers are stated as the last effective dilution, expressed as log to base 2, to show agglutination. The titers are in terms of the titer of the unabsorbed sera; obtained by calculation, with use of the dilution involved in absorption.

logous flagellated strain than on the homologous non-flagellated strain, indicating that the two strains contained a minor common antigenic component. In addition, each serum agglutinated flagellated and non-flagellated suspensions of the heterologous strain to the same end point. These results indicated that the demonstrated common antigen was probably somatic in nature. The possible existence of minor common flagellar antigens was investigated by completely absorbing each serum with the heterologous non-flagellated strain until no agglutinating activity against the common somatic antigen could be detected. Such sera, anti-W 23 absorbed with SB 108 and anti-SB 108-b absorbed with SO 65, were found to possess a low titer of agglutinating activity against the heterologous motile strains, SB 108-b and W-23 respectively. These findings revealed the existence of common antigenic components on the flagella as well as on the cell wall of the two strains.

By analogy with the Kauffmann-White Schema for Salmonella (Kauffmann, 1966a) 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 ..).

The specificities and distribution of the flagellar and somatic antigens were confirmed by absorption and titration of anti-W 23 and anti-SB 108-b with antigenically hybrid strains prepared by transformation. Strain SO 67 was prepared using W 23 as recipient for SB 108-b DNA and selected by the addition of anti-W 23 to semi-solid medium. The strain therefore contained the somatic antigen of W 23 and the flagellar antigen of SB 108-b. The reverse transformation cross and selection produced hybrid strain SO 12, which contained the somatic antigen of SB 108-b and the flagellar antigen of W 23. The suggested antigenic formulae for these strains was:

SO 12 = 1,2:b..

SO 67 = 1,3:a..

The results of serological tests between anti-W 23 and anti-SB 108-b sera and these antigenically hybrid strains are summarized in Table 3 and confirmed the antigenic formulae predicted for the two hybrid strains. Absorption of anti-W 23 (1,3:b..) serum with SO 12 (1,2:b..) removed all the agglutinating activity against SB 108-b or its non-flagellated parent (SB 108) and left a residual agglutinating activity against W 23 and SO 65 (non-flagellated W 23). The residual activity, predicted to be anti-3, was confirmed as being directed against somatic

antigens by its complete removal following absorption with the non-flagellated W 23 strain, SO 65. Similar residual agglutinating activity was revealed following absorption of anti-SB 108-b (1,2:a..) with SO 67 (1,3:a..). The residual activity against flagellated and non-flagellated (SB 108) strains of SB 108-b, presumably anti-somatic antigen activity, was completely removed by absorption with SB 108.

The specificity of the flagellar antigens was also confirmed by absorption. Absorption of anti-W 23 (1,3:b..) with SO 67 (1,3:a..) left a residual agglutinating activity directed only against the flagellar antigen b of W 23 (i.e., activity with W 23 and SO 12), and absorption of anti-SB 108-b (1,2:a..) with SO 12 (1,2:b..) left a residual abblutinating activity only for the flagellar antigen a of SB 108-b (i.e., activity with SB 108-b and SO 67).

This serological study demonstrated the existence of two flagellar antigens in B. subtilis and the possible natural occurrance of allelic forms of the flagellar structural gene.

Phase variation in Bacillus subtilis

Study of the genetic control of flagellation in Salmonella has revealed the existence of genes which on

mutation fix the mutant strain in one phase only of a wild-type diphasic system (Iino, 1964). It was possible that a diphasic flagellar system was present in B. subtilis and that strains W 23 and SB 108-b were fixed in alternative forms of such a system. In Salmonella the alternative phase of mutants fixed in one phase could be isolated by selection in semisolid medium containing antiserum against the manifested antigen. Growth of B. subtilis W 23 in semisolid medium containing anti-b (its expressed flagellar antigen), and of SB 108-b in semisolid medium containing anti-a serum, failed to reveal any strains in which the alternate flagellar antigen was produced, as evidenced by the absence of swarms in either experimental system. This evidence, together with the absence of any data in the literature reporting phase variation in B. subtilis, was taken to indicate, at least as a preliminary hypothesis, the absence of phase variation in B. subtilis. The flagellar antigenic differences between W 23 and SB 108-b were therefore assumed to be expressions of allelic forms of a gene controlling antigenic specificity and, by analogy with the Salmonella system (Iino and Lederberg, 1964), allelic forms of the flagellar structural gene in B. subtilis.

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 serum selection in semisolid medium, transformants with the a antigen could be obtained by exposing competent cells of W 23 (fla$^+$, b antigen) to DNA isolated from SB 108-b (fla$^+$, a antigen). The reverse cross was also performed and SB 108-b transformants with the b antigen of W 23 were obtained. 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. In these studies the DNA preparations were used at concentrations below saturation. These results, summarized in Table 4, indicated that the flagellar antigen was exchanged by transformation in the usual manner. Quantitative comparisons in the reverse test, using W 23 as recipient, could not be performed as W 23 is nutritionally prototrophic and no established auxotrophic mutant strains were available.

Absorption tests (Table 3), previously described, confirmed the flagellar specificity of the transformants and demonstrated that, in both directions of

Table 4

Transformation of strain SB 108-b (*trp*-2$^-$, flagellar antigen *a*) with DNA from strain W 23 (*trp*-2$^+$, flagellar antigen *b*)

Dilution of DNA used	Number of transformants/ ml competent cells		% transformation*	
	flagellar antigen *b*$^+$	*trp*-2$^+$	for *b*$^+$	for *trp*-2$^+$
10^{-2}	2.5×10^5	2.2×10^5	0.17	0.15
10^{-3}	2.8×10^4	3.1×10^4	0.019	0.02
10^{-4}	3.1×10^4	3.0×10^3	0.002	0.002

* % transformation calculated using a determined total viable count of 1.5×10^8 cells/ml.

the cross, the major flagellar antigen of the recipient strain was replaced by that of the donor strain. The involvement of the minor common flagellar antigen could not be ascertained because of the dilution which occurred in the procedure. The somatic antigens were found to have remained unchanged in the transformants analyzed.

As indicated above, *B. subtilis* does not appear to undergo phase variation. Therefore the simplest explanation for these results was that they demonstrated the existence of a locus, termed H by analogy with

Salmonella nomenclature (Iino and Lederberg, 1964), which controlled the flagellar antigen. Accordingly, SB 108-b possessed the H-*a* allele and W 23 the H-*b* allele of the H locus. In *Salmonella* the definition of the H locus has been extrapolated, on the basis of genetic and biochemical evidence, to make H the structural gene for the determination of the primary sequence which determines the tertiary conformation of the component molecules and therefore the antigenic specificity of the intact flagellum. Similarly, the H locus in *B. subtilis* was considered to be the structural gene for flagellin.

Identification of a fla gene

Stocker (1963) demonstrated that non-flagellated mutant strains of *Bacillus subtilis* could be obtained and that they could be transformed to motility by DNA isolated from a flagellated strain. Such mutants were termed *fla*$^-$ mutants. In confirmation of these results, the non-flagellated mutant SO 6 was transformed to motility with DNA preparations from either strain SB 108-b or W 23. With W 23 DNA SO 6 was transformed to motility at the same frequency as it was transformed to *trp*-2$^+$. SO 6 was therefore considered to have a mutation in a *fla* locus, arbitrarily designated *fla*-6.

These results are summarized in Table 5 on page 63 and were placed in that portion of the text because of their relevance to the linkage tests described below.

SO 6, and other selected non-flagellated mutants obtained following ultraviolet irradiation of SB 108-b, were crossed in transformation tests in an attempt to identify additional fla loci. The results are summarized in Table 6 and reveal the presence of four fla loci. SO 6 and SO 8 appeared by these transformation tests to be similar, if not identical mutations.

Table 6

Transformation to motility* of various non-flagellated mutant strains of SB 108-b.

DNA Preparation (i.e., donor)	Competent cells (i.e., recipient) SO 4	SO 6	SO 7	SO 8	SO 9
SO 4	-	+	+	+	+
SO 6	+	-	+	-	+
SO 7	+	+	-	+	+
SO 8	+	-	+	-	+
SO 9	+	+	+	+	-

*"+" indicates the presence of motile transformants, which appeared as swarms in semisolid medium.

Identification of a mot gene

A mutant strain was isolated following ultraviolet irradiation of B. subtilis SB 108-b which did not swarm through semisolid medium and which exhibited no translational motility in broth cultures when viewed under the phase contrast microscope. However, this mutant (designated SO 5) was found to be agglutinated by anti-a flagellar antigen specific sera (i. e., anti-SB 108-b fla$^+$ absorbed with SB 108 fla$^-$) and to possess flagella when examined by direct staining. Such stained preparations demonstrated no morphological differences between the flagella of SO 5 and the wild-type SB 108-b and no differences in number of flagella/organism. On the basis of these findings, the mutant SO 5 was termed "paralyzed" by analogy with similar mutants described in Salmonella (Hirsch, 1947; Friewer and Leifson, 1952; Stocker et al., 1953). The mutation causing such paralysis was described as being in a mot gene (Enomoto, 1966a; Demerec et al., 1966). 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 results of these experiments are summarized in Table 7, which has been placed on page 64 in the text because of its relevance to linkage experiments described below.

The flagella shape mutants of Salmonella (Iino, 1962a, Iino and Mitani, 1966b), of which the curly mutant is the most extensively studied, were reported to possess flagella of abnormal wavelength or amplitude and show a marked inability to exhibit translational motion. The flagella shape mutation was found to be phase specific and inseparable from the H locus. These mutant strains were therefore considered to be a type of H mutant whose flagella shape and ability to move were altered by a change in the primary amino acid sequence of the flagellin molecule. As flagella wavelength determinations on stained preparations on SO 5 were not conclusive, the possibility that SO 5 was due to an H mutation was examined genetically. Separation of the H genes and the presumed mot gene was demonstrated by transformation. SO 12 (SB 108-b given the H antigen b of W 23 by transformation), when treated with DNA from SO 5 (SB 108-b mot$^-$ mutant), yielded transformants possessing paralyzed flagella with the b antigen of the recipient strain unchanged.

The separation of the mot and H genes in B. subtilis demonstrated that the mot$^-$ mutation found in SO 5 was a true mot mutation (Enomoto, 1966a) and not a flagella shape mutant (H mutation). SO 5 was therefore morphologically and genetically a paralyzed mutant

according to the definition of Stocker et al. (1953) and Enomoto (1966a) used for Salmonella strains.

Test for linkage of 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 for the selection of double transformants which were transformed for both fla$^+$ and H antigen b. When such experiments were performed, double transformants were isolated only when the cells were concentrated prior to inoculation and the donor W 23 DNA preparation was used at saturating concentrations. The results are summarized in Table 5 on page 63. At saturating concentrations of DNA double transformants may be obtained which arise from the uptake of two or more separate molecules of DNA. Unlinked genes may incorrectly be considered linked unless the transformations are repeated utilizing several different DNA concentrations. Linkage results are also affected by the method of DNA preparation. The larger the size of the DNA fragments isolated

the more distant will be the linkage relationships that can be measured.

Under the transformation conditions utilized in the present experiments the H and fla-6 loci were considered to be unlinked.

Linkage of the H and mot loci

Linkage between the H and mot loci was tested by transforming SB 108-b mot$^-$ (SO 5 or SO 49) with DNA extracted from strain W 23. Mot$^+$ transformants were selected in semisolid medium containing anti-SB 108-b serum. Double transformants (mot$^+$, H-b) were isolated only when the donor DNA was used at saturating concentrations. The results are summarized in Table 7 on page 64, and were taken to indicate that the H and mot loci were unlinked in the present system.

Test for linkage of the fla-6 and mot loci

Linkage between these two genetic loci could not be tested directly, as mutation in either resulted in nonmotility. Using saturating concentrations of DNA, transformations in both directions were made between SO 5 (mot$^-$) and SO 6 (fla-6$^-$). SB 108-b was also used as a source of DNA. SO 6 was found to yield the same

Table 5

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

Dilution of DNA used	Swarms in semisolid medium containing anti-SB 108-b serum	% transformed for *trp*-2$^+$	% transformed for *fla*-6$^+$	% of *trp*-2$^+$ transformants also transformed for *fla*-6$^+$	% of *fla*-6$^+$ transformants also transformed for *trp*-2$^+$	*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 7

Transformation of SO 5 (SB 108-b *mot*$^-$) with DNA from strain W 23

Dilution of DNA used	Swarms in semisolid medium containing anti-SB 108-b serum	% transformed for *trp*-2$^+$	% transformed for *mot*$^+$	% of *trp*-2$^+$ transformants also transformed for *mot*$^+$	% of *mot*$^+$ transformants also transformed for *trp*-2$^+$	*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

number of *fla*$^+$ transformants with DNA from either SO 5 or SB 108-b. Similarly, SO 5 yielded the same number of *mot*$^+$ transformants with DNA from SO 6 or SB 108-b. In cases of close linkage, the majority of transformants undergo a recombinational event involving both linked genes (Nester *et al.*, 1963). If such close linkage existed here, DNA from a *mot*$^-$, *fla*-6$^+$ donor would yield fewer motile transformants (*mot*$^+$, *fla*-6$^+$) from a *mot*$^+$, *fla*-6$^-$ recipient than would DNA from a *mot*$^+$, *fla*-6$^+$ donor, because the majority of *fla*$^+$ transformants would also incorporate the *mot*$^-$ gene of the donor. The reverse cross involving a *mot*$^-$, *fla*-6$^+$ recipient would show similar inequality in the number of transformants when different donor DNA's were used. As such a difference in the number of transformants was not observed experimentally when different DNA preparations were used, it was concluded that the *mot* and *fla*-6 loci were not closely linked.

Tests for linkage of the mot, H, and fla genes to the trp-2 gene

In separate experiments, competent cells of SO 5 (*mot*$^-$, H-*a*) and SO 6 (*fla*-6$^-$, H-*a*) were exposed to DNA from W 23 (H-*b*). Motile transformants were tested for

their tryptophan requirement and tryptophan transformants were tested for their ability to swarm in semisolid medium. Double transformants of the trp-2^+, mot$^+$ and trp-2^+, fla-6^+ types occurred very rarely. The values obtained for the cotransfer index in both cases was 0.01. The rarity of double transformants and the low cotransfer index were reflective of unlinked markers.

Double transformants of the trp-2^+, H-b types were also extremely rare and did not indicate linkage between the two markers.

By the tests described above, utilizing DNA prepared as described in the "Methods" section, the flagellar genes (mot, fla-6, H) were not found to be linked to one another, and none was found to be linked to the trp-2 locus.

Transformation with nonmotility markers used as donors

Competent cells of SO 14, and SB 108b 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. Colonies appearing non-motile in semisolid medium were tested

with the DNA used in their preparation to ensure genetic identity of their fla (or mot) gene with that of the donor. The results of these experiments are summarized in Table 8. 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 transforma- to trp-2$^+$ at 1.2%; in all cases, the DNA solutions were used at concentrations above the saturation. This finding was significant for an understanding of the fate of the competent cell after transformation and will be discussed later.

B. Adsorption specificity of bacteriophage PBS1

Joys (1965) demonstrated that phage PBS1 did not form plaques on a non-flagellated derivative (SB 108) of B. subtilis strain 168 because of the failure of the strain to adsorb the phage. He pointed out the analogy between this behavior and the properties of the known flagellotrophic phage chi for Salmonella (Meynell, 1961) and suggested

Table 8

Transformation of strain SO 14 to non-motility and identification of non-motile transformants isolated

DNA preparation	Number of colonies tested	Number of non-motile colonies isolated	Transformation to motility of isolated non-motile transformants with DNA preparation from:		
			SB 108-b	SO 6	SO 5
SO 6 (*fla*-6$^-$)	940	6	+	-	+
SO 5 (*mot*$^-$)	960	3	+	+	-

that phage PBS1 might be a second example of a flagellotrophic phage. With the isolation of various mutants affected in their flagellation properties, particularly the paralyzed mutant SO 5, it was possible to test the validity of this suggestion by examining the adsorption characteristics of phage PBS1 in greater detail.

Four non-flagellated strains (SO 4, SO 6, SO 7, and SO 9) and the paralyzed strain (SO 5) were used as indicators in a plaque assay of a lysate of PBS1-V1 together with flagellated and motile strain SB 108-b.

Phage PBS1-V1 failed to produce plaques on any of the flagellaless mutants or on the paralyzed strain SO 5 even when 10^8 infective units (multiplicity of infection = 1.0) were present in the overlay.

Adsorption tests indicated that the failure to produce infective centers reflected the inability of the phage to adsorb to the particular strain being tested. The results are presented in Table 9. All the nonflagellate strains and the paralyzed strain, SO 5, failed to adsorb any phage under the present conditions, in which the motile strain, SB 108-b, adsorbed 98% of the infectious

Table 9

Adsorption of phage PBS1-V1 with motile and non-motile strains of *Bacillus subtilis* and with preparations of isolated flagella

Adsorbing suspension	Flagellation	Motility	Titer of phage after adsorption	% adsorption
SB 108-b	++	++	3×10^4	98
SO 4	-	-	2×10^6	0
SO 5	++	-	2×10^6	0
SO 6	-	-	2×10^6	0
SO 7	-	-	2×10^6	0
SO 9	-	-	2×10^6	0
SB 108	-	-	2×10^6	0
SB 108-b blender supernatant fluid	/	/	2×10^6	0
SB 108-b partially purified flagella	/	/	2×10^6	0

phage particles.

Table 9 also shows the results obtained when isolated and partially purified flagella preparations were mixed with the phage; no inactivation of phage could be demonstrated.

The results indicate that phage PBS1-V1 specifically adsorbs to functional flagella of B. subtilis strains. The receptor site for the phage would thus seem to be on or closely related to the flagella. The functional state of the flagella was also shown to be important; detached flagella and flagella attached but paralyzed (nonfunctional in locomotion) failed to adsorb the phage.

Meynell (1961) reported that mixing a suspension of a susceptible Salmonella serotype with a lysate of the chi-phage resulted in the rapid cessation of motility followed by the clumping of cells of certain serotypes. The immobilization effect was also noted for phage PBS1-V1, but no clumping was observed in this system. Addition of PBS1-V1 to an actively motile culture of SB 108-b, at a multiplicity of 10, resulted in the rapid cessation of motility of SB 108-b. Observations under the phase-contrast microscope

revealed that the cells of SB 108-b became completely non-motile within 2 to 3 min following the addition of phage. The adsorption of phage thus renders the flagella nonfunctional.

DISCUSSION

A. Genetic Control of Flagellation

The results presented demonstrate the existence of three separate, distinct loci controlling flagellation in *Bacillus subtilis*. They also represent the first description of a serological analysis of this genus. As in *Salmonella* there were found *fla*, *mot*, and H loci concerned with the presence or absence of flagella, the ability of the flagella to function in motility, and the primary amino acid sequence determining the antigenicity and morphology of the flagella. The *mot* mutation was also studied for its role in the resistance to phage PBS1 exhibited by the paralyzed mutant.

The ability to replace by transformation the flagellar antigen of SB 108-b with that of W 23, and vice versa, was most simply explained as the replacement of a gene specifying the flagellar antigen by its allele. Studies with *Salmonella* (Iino, 1964) have demonstrated that, with certain monophasic mutants of a wild-type diphasic system, transfer of genes other than H genes can result in the production of a different flagellar antigen in the recipient. The explanation proposed above for *B. subtilis* was based upon the fact that phase variation has not been demonstrated in this species; no swarms were observed when

SB 108-b or W 23 were inoculated into semisolid medium containing anti-a of anti-b serum respectively. By analogy with the terminology used for Salmonella (Lederberg and Iino, 1956), the structural gene for the flagellar antigen was termed H. Recently it was suggested (Demerec et al., 1966) that "genes determining and regulating the synthesis of flagellar antigens" be termed hag, in which case the structural gene would become hag H. As only one type of flagellin molecule and was not limited to the genetic material specifying that region of flagellin which is of antigenic importance. B. subtilis strains SB 108-b and W 23 also appear to produce one type of flagellin molecule for a given flagellum. Purified flagellin preparations of each strain were found to produce a single band in disc polyacrylamide gel electrophoresis (Joys and Frankel, 1967). A similar definition of H as the structural gene for the whole flagellin molecule was therefore suggested for B. subtilis. Two cases can be cited where this broader definition proves justified. Both are examples in which H mutation resulted in alteration of the primary amino acid sequence of the flagellin molecule without altering its serological specificity. The curly mutant of Salmonella possessed flagella whose wavelength measured one-half that found in the wild-type organism but was antigenically identical to the wild-type. Tryptic

peptide analysis revealed a single peptide difference between curly and wild-type flagellin (i.e., alteration in the primary amino acid sequence) (Enomoto and Iino, 1966) and genetic analysis placed the mutation within the H locus (Joys and Stocker, 1963). The straight mutant of Salmonella (Iino and Mitani, 1967b) possessed straight flagella which were found to be serologically identical to wild-type. The mutation was phase-specific. A similar mutant has been described in B. subtilis and shown to possess flagella which lack the long period helix (Raimondo, Lundh, and Martinez, 1968). Biochemical studies of this strain revealed a dipeptide difference between the mutant and the wild-type flagellin. A single amino acid substitution of alanine for valine was found to have occurred in the straight flagellar mutant (Martinez, Ichiki, Lundh, and Tronick, 1968). Detailed genetic analysis has not yet been reported for the straignt flagellar mutant of either Salmonella or Bacillus, but based on the biochemical evidence in B. subtilis and the ability to cotransduce the mutation with the H locus in Salmonella, the mutation will most probably be found to map within the H locus.

Additional research has also been reported on the mapping of antigenic specificity-determining sections

within the H-1 locus by the establishment of a system for selecting intra-H-1 recombinants (Yamaguchi and Iino, 1969). P22 phage-mediated transductions were carried out between pairs of H-1 linked *fla*$^-$ mutants, whose H-1 alleles were different and whose *fla*$^-$ sites were on opposite sides of H-1. The *fla*$^+$ transductants were isolated and the composition of their flagellar antigens examined with specific antisera. Flagellin of a serologically defined antigen recombinant was proved to be a recombinant of the two-pareatal flagellins by fingerprint analysis of tryptic digests. Summarizing the relative positions inferred from an analysis of the antigen recombinants it was shown that each of the antigenic specificity-determining sections maps as a unit; and that as a whole they form a linear array within H-1. Sections specifying some tryptic peptides of flagellins were also mapped within H-1 (Yamaguchi and Iino, 1969).

Transformation studies permit an examination of closely linked markers (Takahashi, 1966) and by this method the three loci controlling flagellation in *B. subtilis* were found to be unlinked to one another and each unlinked to the trp_2 locus. Linkage to the trp_2 locus was examined because of the reported linkage in *Salmonella* between the *trp* loci H-1, *mot*, and several

fla loci as measured by cotransduction (Smith & Stocker, 1962; Enomoto, 1966b). Transductional tests reveal more distant linkages than do transformation analyses, as a larger fragment of the chromosome is transferred in transduction (Takahashi, 1966), which might explain the different results in linkage studies in *Salmonella* and *Bacillus*.

Detailed genetic analysis of the flagellar genes of *Escherichia coli* has recently been reported (Armstrong and Adler, 1967; Armstrong and Adler, 1969). H, *mot*, and *fla* genes have been demonstrated (Furness, 1958; Armstrong and Adler, 1967; Ørskov and Ørskov, 1962) and shown to have a chromosomal location similar to those reported for *Salmonella* (Armstrong and Adler, 1969). However, *E. coli* was reported not to exhibit phase variation and to possess only one H locus (Furness, 1958; Matsumoto and Tazaki, 1967). Crosses between *Salmonella* Hfr and *E. coli* F^- demonstrated that the H-1 locus of the former was allelic to the single H locus of the latter (Mäkelä, 1964). Tests of allelism are not possible between *B. subtilis* and either *Salmonella* or *E. coli* but other methods of testing for linkage relationships and chromosomal location of markers might reveal genetic similarity between the genes controlling flagellation in the difference genera. Tryptic peptide analyses of serologic

and morphologic mutants should provide insight into biochemical similarities of the different flagellins from the different genera.

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 competent cell following transformation. Nester and Stocker (1963) demonstrated that in colonies resulting from the growth of individual transformed cells, 75-90% of the cells were of the recipient genotype. This finding was attributed to the transformation of only one of the two nuclei present in the competent cell at the time of DNA uptake. Their results would predict that a colony arising from a cell transformed with a marker conferring non-motility would contain mainly motile progency 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 following 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 progency 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%, at least for these markers. Recently, F. E. Young (personal communication) showed that transforming DNA became attached to the forespore nucleus, suggesting that this was the nucleus which contributed to the transformed progeny. Nester and Stocker (1963) induced the competent state by a method different from the one used in the present experiments (Anagnostopolous & Spizizen, 1961), and it is possible that the discrepancy between their results and those described was due to different physiological conditions employed.

Several methods have been described 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 chromosones (Dubnau, Goldthwaite, Smith, and Marmur, 1967). These approaches as well as data from transductions mediated by phage PBS1 would be of interest as a means to detect linkage between genes controlling flagellation and other loci.

B. Adsorption Specificity of Phage PBS1

The results of this investigation confirmed the suggestion that the receptor site for phage PBS1 was located on the flagella. The adsorption specificity of PBS1 was shown to be similar to that of the only other

reported flagellotrophic phage, chi-phage (Meynell, 1961), in that functional flagella were required for irreversible adsorption. Phage were not adsorbed by non-flagellated strains, detached flagella, or paralyzed strains (organisms with morphologically and antigenically wild-type flagella which were non-motile in liquid and semisolid medium). The term "functional flagella" was used here to denote flagella of organisms able to move through semisolid medium or exhibit translational motility in liquid medium. In light of experiments discussed below motility may not be the only reflection of the functional state of the flagella.

The adsorption specificity and host range of chi-phage have recently been studied in an attempt to define the processes of attachment and infection by the phage. Chi-phage has been shown to attack flagellated motile strains of Serratia marcescens (Iino and Mitani, 1967a) and Escherichia coli (Schade and Adler, 1967). It has been purified from infected E. coli and found to possess 46% deoxyribonucleic acid (DNA) and 54% protein, based on the Dische method (Dische, 1955) and total nitrogen analysis (Schade and Adler, 1967). The purified DNA exhibited a melting curve characteristic of double-stranded DNA and based on length measurements was estimated to have a molecular weight of 42 million (Schade and

Adler, 1967). A host range mutant of chi, termed M8, was found to attack Salmonella strains which possessed antigens of the g-complex (Sasaki, 1962 cited in Yamaguchi, 1968). The sensitivity to infection by M8 differed for different serotypes of the g-complex, but was shown by transduction to have resulted from factors other than flagellar antigenic specificity. These factors were suggested to control the infection process of the phage after its adsorption to a susceptible host (Yamaguchi, 1968).

Early electron micrographs (Meynell, 1961) of chi-phage and its attachment to susceptible bacteria confirmed the suggestion that the receptor site for chi-phage was located on the flagella. No phage were seen on the bacterial surface even at the bases of the flagella. More recent time-lapse electron micrographs of negatively stained preparations (Schade, Adler, and Ris, 1967) indicated that under conditions of high multiplicities of infection the phage can first be seen to be attached by its tail fiber to the filament of the flagellum and later similarly attached to the base of the flagellum. The phage at the base were often empty (i.e., had injected their DNA), whereas no empty phage were found on the flagella filaments. The suggested sequence leading to infection was therefore postulated as the adsorption of the phage to one of many sites along the flagellum filament,

the movement of the phage to the base of the flagellum, followed by the injection of the phage DNA into the bacterium in the area at the base of the flagellum (Schade et al., 1967). Motility of the flagella would, therefore, play a role in increasing the likelihood of the initial adsorption, or the transfer of the phage to the flagellar base, or both. The use of high multiplicities (100 and 500 phage/bacterium) raises the question as to whether this postulated sequence is the actual process which occurs under more natural conditions where the multiplicity of infection is very much lower. High multiplicities were also used for the electron microscopic observation of phage adsorption to flagellar filaments and bases of bacteria whose motility had been strongly inhibited by exposure to cold or anaerobic conditions. The finding of phage at both locations under both conditions of artificial paralysis led the authors to suggest that motility was not essential; random collisions could occur between the phage and the flagellar filaments and bases. However, bacteria with genetically paralyzed flagella were shown to be resistant to phage infection (Schade et al., 1967). Even at high multiplicities of infection there were only a small number of phage seen attached to the filament and even fewer found at the base of the flagella. Those at the base were never

found to have injected their DNA under conditions where mostly "ghosts" (phage heads empty of their DNA) were seen in preparations of phage and motile flagellated bacteria. The authors (Schade et al., 1967) suggested on the basis of these findings that both the bases and the filaments of the flagella of genetically paralyzed mutants were abnormal for phage attachment. Uranyl acetate was used as the stain for electron microscope preparations when it was necessary to locate all phage on the bacteria as phosphotungstic acid was found to obscure the bacterial surface (Schade et al., 1967). The use of phosphotungstic acid may explain the earlier failure (Meynell, 1961) to observe chi-phage associated with the bacterial surface at the base of the flagella. One would anticipate that the processes of attachment and infection of chi-phage to its susceptible host would be the same for different bacterial genera.

Phage PBS1 has also been further studied and investigations into its purification, structure and attachment site have been reported (Eiserling, 1967; Raimondo et al., 1968). PBS1 was also found to be a DNA-containing virus but larger in size and more complex structurally than chi-phage. The molecular weight of the phage-specific DNA was reported to be 2×10^8 (Hunter, Yamagishi, and Takahashi, 1967) as compared with 4×10^7 for chi-phage

(Schade and Adler, 1967; incorrectly cited as 4 X 10^6 in Raimondo et al., 1968). Negative staining revealed head profiles consistent with icosahedral symmetry for both PBS1 and chi-phage (Eiserling, 1967; Schade and Adler, 1967). Besides being larger in size than chi-phage, two unique structures were observed for purified PBS1 (Eiserling, 1967). Three large helical tail fibers were seen attached to a base plate at the distal end of the tail of PBS1. They might correspond to the single large tail fiber found in chi-phage which appears as an extension of one prong of the two-pronged tapered tail (Schade and Adler, 1967). The second structure reported for PBS1 was a number of thin fibers which project outward from the base of contracted tail fiber sheaths. These fibers were not seen on uncontracted phages; they were termed "contraction fibers" to distinguish them from the tail fibers of other phages (Eiserling, 1967). Chi-phage was reported to be non-contractile (Bradley, 1967) and such thin fibers have never been observed.

Electron microscopic observation of uranyl acetate stained preparations revealed the attachment of PBS1 along the length of the flagella of motile B. subtilis by means of the helical tail fibers of the phage (Raimondo et al., 1968). Rarely, if ever, were phage seen attached to the cell body, especially if a multiplicity of infec-

tion of less than 10 was used. The different results reported for PBS1 and chi-phage, as to the finding of phage at the base of the flagella as well as along the filament for chi but not for PBS1, was probably a reflection of the different multiplicity of infection used rather than a fundamental difference in the process of attachment and infection of the two phages.

PBS1 was also reported (Raimondo et al., 1968) to be adsorbed by cyanide-treated bacteria as well as to protoplasts of flagellated bacteria. The addition of KCN to the medium or the protoplasting of bacteria resulted in flagella which were unable to function in motility (i.e., organism and protoplast were non-motile) but still capable of adsorbing phage PBS1. One mutant strain of B. subtilis was isolated which possessed flagella but was non-motile in liquid and semisolid medium. Both adsorption and infection by PBS1 to this mutant were essentially the same as for the wild-type (Raimondo et al., 1968). The flagella of this strain lacked the long period helix, and on biochemical analysis its purified flagellin was shown to have the amino acid alanine substituted for the amino acid valine found in the wild-type flagellin (Martinez et al., 1968). This strain was incorrectly described as a paralyzed mutant (Raimondo et al., 1968; Martinez et al., 1968). This designation

was improper on genetic, morphological and biochemical grounds. The mutant was not shown to possess a mutation in a *mot* gene as do true genetically paralyzed strains (Enomoto, 1966a; Enomoto, 1966b; Frankel and Joys, 1967), but rather the finding of an amino acid substitution in the primary sequence of its flagellin indicated that it was most probably an H mutation. The alteration in primary amino acid sequence in the mutant has in turn changed the morphological appearance and ability of the mutant flagella to move; true genetically paralyzed mutants revealed no morphological alteration. This mutant, whose flagella lack the long period helix, was more similar to the curly mutant whose mutation maps within the H locus. The curly mutant, although abnormal in its flagellar morphology and motility, also showed a partial adsorption of *chi*-phage (Schade *et al.*, 1967). It would, therefore, seem appropriate to conclude that both *chi*-phage and PBS1 do not adsorb to genetically paralyzed mutant strains and that the absence of motility *per se* was not the sole criterion for the resistance exhibited by these strains.

Artifically paralyzed strains, shown to be partially or fully sensitive to phage infection, were perhaps non-motile for a very different reason than were the genetically paralyzed strains. The product of the wild-type *mot* gene would presumably be produced in the artifically

paralyzed strains. The actual function of the mot gene has not been determined and its product may affect characters other than motility. The phage resistance of mot strains might also only be a secondary result of the mutation and might not be primarily a result of visual non-motility. Alternatively, in curly mutants, straight flagellar mutants, and in artifically paralyzed organisms the flagella per se may have retained motility, but this movement may not be capable of effecting locomotion of the bacterial bodies.

Future investigations of many more genetically paralyzed strains are necessary to clarify the nature of the product and effects of the mot gene and its relationship to motility and phage susceptibility. As mot is genetically distinct from H, the structural gene of the flagellin, the flagella per se should not be the target of investigation but instead its activation, functioning, regeneration, and basal attachment site.

Phage infection can be viewed as a multistep process: adsorption of the phage to a susceptible strain, migration of phage, injection and penetration of DNA, production of mature phage. A susceptible strain is one which possesses flagella of the proper antigenic type (implying proper charge and conformation resulting from the primary amino acid sequence) and functional state (not always

definable by visible motility). Other factors may then affect the ability of the phage to penetrate and to mature within the organism.

SUMMARY AND CONCLUSIONS

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 genetic control of flagellation in B. subtilis was found to be similar to that described for Salmonella, with the exception that in B. subtilis no antigenic phase variation could be detected and the three loci were unlinked in transformation tests. The serological analysis of B. subtilis was the first reported for this genus, and revealed that strains W 23 and SB 108-b, a derivative of 168, differed in their major flagellar antigen.

The mutants isolated in the course of the genetic analysis of B. subtilis provided strains which were studied for their ability to adsorb bacteriophage PBS1. By the use of newly isolated nonflagellate mutants, the location of the receptor site for phage PBS1 was confirmed as being on the flagella of B. subtilis. The functional state of the flagella was also shown to be important for susceptibility; partially purified flagella isolated from a culture of

susceptible organisms and a genetically paralyzed mutant (possessing morphologically normal flagella, but non-motile) failed to adsorb the phage. This flagellotrophic phage was therefore shown to be similar in the location of its receptor site and adsorption specificity to the only other reported flagellotrophic phage, bacteriophage-chi, which attacks susceptible strains of Salmonella, Escherichia coli and Serratia marcescens.

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