GENETIC STUDIES OF PARALYZED MUTANTS IN SALMONELLA.

I. GENETIC FINE STRUCTURE OF THE *mot* LOCI IN *SALMONELLA TYPHIMURIUM*¹

MASATOSHI ENOMOTO

National Institute of Genetics, Misima, Japan

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THE structure of bacterial flagella has been extensively studied with the advancement of electron microscopy and protein chemistry. However, questions on the function of flagella as locomotor organs, such as the propulsive mechanism and the energy-supplying system, remain unanswered. This is mainly due to the limitations of available experimental techniques. One method used for attacking the fundamental problems of flagellar movement is a genetic approach with paralyzed mutants, that is mutants which though flagellated are nonmotile. Flagella of the paralyzed mutant do not differ from those of the wild-type motile cell in either antigenicity, number per bacterium, shape under electron microscope (ENOMOTO, unpublished data), or configuration by X-ray diffraction (BEIGHTON, PORTER and STOCKER 1958). Both the wild type and the paralyzed mutants show flagellar antigenic phase variation (ENOMOTO, unpublished data). These facts suggest that the paralysis is not due to any defect of flagella themselves, but to a defect of the flagellum-activating mechanism within the bacteria.

Preliminary genetic analysis of the paralyzed mutants of *Salmonella typhimurium* was reported by STOCKER, ZINDER and LEDERBERG (1953) and IINO (1958). STOCKER *et al.* showed that motility of a paralyzed mutant could be restored by transduction from the wild type or from another paralyzed mutant. IINO found by transduction experiments with four paralyzed mutants that their mutational sites were contained within a single functional (complementation) unit. For the present investigation, nearly 100 paralyzed mutants were isolated from *S. typhimurium*. A genetic fine-structure analysis of the motility loci has been carried out by means of complementation, deletion, and two-factor transduction tests.

MATERIALS AND METHODS

Nomenclature: The mutant gene causing paralysis of flagella was designated *mot*, for motility (IINO 1958). The strain number for the mutants was assigned mechanically according to the order of isolation, and the mutant number was assigned from 201 on for the paralyzed mutants derived from *S. typhimurium*, TM2. When mutational sites were grouped by complementation tests, a capital letter designating the functional unit was inserted between the gene symbol, *mot*, and the mutant number (e.g. *motA-201*). The mutant number was also used to designate the corresponding mutational site.

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Organisms: All of the mutants used in this work were derived from a wild-type strain of *Salmonella typhimurium*, TM2 (strain LT-2 of ZINDER and LEDERBERG 1952). Almost all of the paralyzed mutants were isolated by a screening method consisting of a combination of semisolid medium and the "motile-specific" *chi* phage (MEYNELL 1961; ENOMOTO and IINO, 1963). The paralyzed mutants from SJ597 (*mot*-269) to SJ637 (*mot*-300) were isolated by induction with ultraviolet light; others were of spontaneous origin. Strain SJ697, in which *H1i* is replaced by *H1gp* of *S. dublin*, was used for the test of joint transduction of *mot* and *H1*. Histidine-requiring mutants were obtained from Dr. P. E. HARTMAN, Johns Hopkins University, Maryland. Phage P22 was used for transduction (ZINDER and LEDERBERG 1952). Phage suspensions were prepared as described by STOCKER *et al.* (1953), and assayed by a modified agar-layer technique (ADAMS 1959).

Media: Nutrient broth medium (pH 7.2) was composed of 1% peptone (Kyokuto) and 1% meat extract (Mikuni). Nutrient agar (NA) medium contained 1.5% agar in addition to the nutrient broth. Semisolid nutrient gelatin agar (NGA) medium was prepared by the addition of 0.4% agar and 8% gelatin to the broth medium. The composition of Davis minimal medium (MM) and complete EMB medium was described by LEDERBERG (1950).

Observation of abortive transduction for complementation test: One volume of an overnight broth culture of a paralyzed mutant (approximately 1×10^9 cells per ml) was mixed with an equal volume of phage suspension propagated on a donor strain (approximately 5 to 10×10^9 plaque forming units per ml), and 0.1 ml of the mixture was inoculated on a NGA plate. After incubation for 15 to 18 hours at 37°C, the plate was examined for the presence of trails under the low-power binocular microscope. In transduction of motility, trails appear if the partial heterozygote produced by abortive transduction has the wild-type, motile, phenotype. A *mot*-recipient will therefore give trails in transductional crosses with a wild-type donor if its *mot*-allele is recessive to *mot*⁺; and also in crosses with a nonidentical *mot*-mutant if the two *mot*-mutants can complement one another (LEDERBERG 1956; STOCKER 1956). The number of trails produced in crosses between *mot*-mutants was examined semiquantitatively and divided into three grades: 100–50%, 50–1%, and less than 1% of the number observed in the control crosses of *mot*- with the wild-type donor.

Identification of deletion mutants: The paralyzed mutants were incubated on NGA medium overnight at 37°C to identify unstable mutants. Approximately 1×10^9 cells of the stable mutants were further incubated on NGA medium for three days. Among the mutant strains producing no swarms after this incubation, those which failed to produce swarms by transduction from more than two other mutants were regarded as deletions.

Mapping by recombination frequencies in a two-factor cross: 0.1 ml of the mixture of equal volumes of bacterial and phage suspensions (1×10^8 cells, 5×10^8 plaque forming units per ml) was streaked on each NGA plate. When phages prepared from the wild-type bacteria were used, the mixture was diluted 50-fold after 10 minutes adsorption and streaked. After about 8 hours incubation at 37°C, the number of swarms was counted. Recombination frequency was expressed as a fraction percent of the number of swarms per 10^8 recipient cells treated with phage grown on a given *mot*-donor to that treated with phage grown on the wild-type donor. In order to obtain the mutually comparable recombination frequencies, one series of transduction tests was carried out simultaneously with the same recipient culture. At least ten platings were made for each mixture as well as control platings with uninfected bacteria: when revertants appeared, their number was subtracted from that of the motile recombinants. The mean recombination frequencies of two separate experiments were used for the mapping.

RESULTS

Grouping of paralyzed mutants by complementation tests: Initially, 30 mutants chosen at random were examined in all pairwise combinations for abortive transduction. All the mutants produced trails in transduction tests with the wild-type donor. By the grouping of noncomplementary mutants, three complementation

groups were established. Representative mutants of each group were further used for grouping the remaining mutants. With 15 exceptions, 97 paralyzed mutants were classified as follows: 52 in *motA*; 27 in *motB*; and 3 in *motC*. Of the remaining 15 mutants, *mot*-292 complemented only *motC* mutants and was found in later experiments to be a long deletion. The other 14 mutants complemented *motC* mutants normally. However, they produced weak responses with the representative mutants of either *motA* or *motB*: the number of trails was less than 50% of that in the control crosses. Some of these mutants showed weak responses with both *motA* and *B* mutants. In the "weak response," the decrease in the number of trails was often accompanied by the decrease in the number of minute colonies forming each trail. The similar phenomenon was also observed in partial complementation of *fla*⁻ mutants (INO and ENOMOTO 1966). In the partial complementation, however, the mutant which produced weak responses with mutants of the same group showed normal responses with other groups. In order to distinguish from partial-complementing mutants, these 14 mutants are, henceforth, designated as weak-complementing mutants. The complementation pattern obtained from the semiquantitative experiments among 40 representative mutants including the above 14 mutants is shown in Figure 1: mutants from *mot*-257 to 246 belong to *motA*; 295 to 211 belong to *motB*; 244 to 279 to *motC*; and 297 to 262 are weak-complementing mutants. The mutants in each of the three groups complemented all members of other two groups, but did not complement the mutants in the same group, except that they showed partial complementation in some combinations of the same group: less than 1% as compared with the frequency obtained in crosses with the mutants of other groups. One mutant, *C*-272, when used as a recipient, did not either complement or only complemented weakly *motA* and *B* mutants, whereas as a donor normal complementation with *motA* and *B* mutants occurred. The weak response was also observed in transduction from the wild-type donor. *C*-272 seems to carry a factor which suppresses trail production. The 14 weak-complementing mutants could be tentatively subdivided into two classes, *motA* (12 mutants) and *motB* (2: 227, 262), from the following observations: a difference in relative number of trails was observed between the crosses with *motA* and *B* mutants; *motB*211 was found to complement normally most of these *motA* mutants; and *mot*-227 and -262 were found to be overlapped by the long deletion *B*-292. The weak-complementing *motA* mutants also showed weak responses with *B*-272 and *B*-262, and with each other in certain combinations.

Transduction between *motA* and *motB* mutants always yielded a small number of swarms (wild-type recombinants) as compared with the transductions from the wild-type or *motC* mutants. This behavior can be explained by assuming the two *mot* loci *A* and *B* are jointly transduced: the mutant allele *motA*⁻ is transduced into a recipient *motB* mutant together with the wild-type allele *motB*⁺ and vice versa.

Phenotypic character of the mot mutants: The mutant strains carrying allele of the three *mot* genes were phenotypically similar: they were indistinguishable from each other by means of growth tests in minimal medium, flagellar mor-

tional unit, and transductions were carried out from motile auxotrophic or non-sugar-fermenting mutants to these *mot* mutants on NGA medium. Approximately 100 swarms were picked from each cross and streaked on MM or EMB-sugar media. None of the auxotrophic and sugar markers tested (*proB*, *gal*, *tryA*, *phe*, *gly*, *cysC*, *ser*, *argE*, *mal*, *isl*, and *thr*) was transduced jointly with *mot*. In one experiment, *hisA* was transduced with *motA*-229 at a frequency of approximately 3%; however, there was no reproducibility. In order to strengthen this conclusion, the transductions from *hisE*-11 and *hisG*-46, which are mutants in the end cistrons of the *his* cluster (HARTMAN, LOPER, and ŠERMAN 1960), to *motA*-229 were carried out, but the results were negative.

Joint transductions with *H* genes, the structural genes of flagellin (INO and LEDERBERG 1964), were also tested. Swarms produced in the transduction from SJ697 (*mot*⁺, *H1gp*) to the paralyzed mutants (*mot*⁻, *H1i*) were streaked on NA medium and after growing on the medium their H-antigens were typed by slide agglutination tests with anti-*gp* and anti-*i* serum. In the crosses with *A*-229 and *B*-231, only the motility character of the donor was transduced, but in the cross with *C*-224, *H1* and *mot* were transduced jointly at a high frequency. In Table 1, the frequencies of joint transductions between *H1* and three *motC* are shown. The three mutational sites of *motC* were inferred to be arranged in the sequence: *C*-279-272-224-*H1*.

Mapping with deletion mutants: Ten deletion mutants were found from *motA* and two from *motB*, defined as described in MATERIALS AND METHODS; eight of them had been obtained by induction with ultraviolet light. Transduction tests in all possible combinations among the ten *motA* mutants showed that eight mutants failed to give motile recombinants with one or more mutants, while the remaining two (*A*-294 and -297) recombined with all other mutants. By virtue of concordant overlaps, the eight mutants were arranged in a linear sequence (Figure 2). In order to map the more unstable mutants, qualitative tests for the presence or absence of recombination were carried out between the ten mutants and all other members of *motA*. To avoid confusion between recombination and reverse mutation, the deletions were used as recipients and the reciprocal tests were performed only in crosses with relatively stable mutants. Of the 54 mutants tested, 15 gave no motile recombinants with at least one of the deletions. *A*-294 and -297 also overlapped some reverting mutants. *A*-294, though it only overlapped one reverting mutant *A*-214, was regarded as a deletion, because in the

TABLE 1
Frequency of joint transduction between motC and H1

Recipient	No. of swarms tested	Swarms with <i>i</i> -antigen	Swarms with <i>gp</i> -antigen	Percent of joint transduction	Control without phage
<i>C</i> -224	90	56	34	37.8	0
<i>C</i> -272	108	74	34	31.5	0
<i>C</i> -279	120	100	20	16.7	0

Transductions were carried out from SJ697 (*mot*⁺ *H1gp*) to *motC* mutants (*H1i*) on NGA medium. Swarms were streaked on NA medium, and their H antigens were typed by slide agglutination tests with anti-*gp* and anti-*i* serum.

FIGURE 2.—Genetic map of *motA*. Mutants described vertically are deletions and the extents of their deletions are indicated as thick horizontal bars in which the top eight form one deleted region with overlaps; the mutants lettered horizontally are reverting mutants inferred to be mutated in the deleted regions indicated from their failure to give wild-type recombinants with same deletion mutants. Deletion mutants A-294 and -297 and reverting mutant A-234 are weak-complementing mutants.

cross between A-214 and a reverting mutant A-245 swarms were produced at much higher frequency than that in the cross between A-294 and A-245.

Out of two deletions of *motB* (290 and 292), B292 failed to give motile recombinants with all 29 *B* mutants and with two mutants of *motA*: A-246 and A-300. B-292 was regarded as a long deletion covering all of *motB* and a part of *motA*. It is suggested that *motA* and *motB* form contiguous functional units, and that A-246 and A-300 are located most closely to the *motB*. B-290 overlapped reverting mutants B222 and -225.

Deletions A-238, -277, -282, -294, and -297 had been used for the complementation tests. Except for A-294 and -297 which were weak-complementing mutants, they always complemented the mutants belonging to other functional units.

Mapping by recombination frequencies in two-factor crosses: The mutational sites of A-246 and A-300 which are overlapped by deletion B-292 were initially mapped by recombination tests with *B* mutants. The recombination frequencies were calculated by taking the number of motile recombinants recovered in the cross with the wild-type donor as 100%, and corrected by phage titres. The recombination frequencies in transduction from A-246 and A-300 to B-261 were 2.75 and 3.82% respectively, and to B-275 were 4.83 and 5.63%. A-246 was inferred to be located nearer to the *B* unit than A-300. The following transductions were performed with A-246 as a recipient and the mutants used for the complementation tests (Figure 1) as donors. Three series of transductions were carried out: from 11 *motA* mutants to A-246; from 11 *motB* mutants to A-246; and from 14 weak-complementing mutants to A-246.

In Table 2, the results of transduction tests between the *motA* mutants and A-246 are shown. The recombination frequencies ranging from 0.19% (A-252) to 1.74% (A-257) were obtained. The deletion mutants, A-238, -277, and -282 showed recombination frequencies of 0.61, 0.74, and 0.94% respectively. As shown in Figure 2, the eight deletion regions from A-238 to A-282 overlapped to indicate a long deleted region. Therefore, the mutants showing recombination frequencies from 0.61 to 0.94% should be overlapped by some of the eight dele-

TABLE 2
Recombination frequency in transduction from motA mutants to A-246

	Mutant No.											TM2
	252	259	238 [†]	266	267	277 [†]	229	282 [†]	269	240	257	
Phage titre (%)	319.5	94.4	166.7	123.6	116.7	166.7	138.9	88.9	126.3	94.4	83.3	100
Recombination frequency	0.61	0.39	1.02	0.79	0.82	1.23	1.05	0.83	1.54	1.38	1.44	100
Recomb. freq. corrected*	0.19	0.42	0.61	0.64	0.71	0.74	0.76	0.94	1.22	1.46	1.74	100

* Corrections were carried out to make the phage titre 100.

[†] Deletion mutant.

TABLE 3
Recombination frequency in transduction from motB mutants to A-246

	Mutant No.											TM2
	215	296	225	239	261	231	295	283	247	275	211	
Phage titre (%)	118.0	43.1	180.0	102.8	105.5	97.2	166.5	73.6	63.9	66.6	166.5	100
Recombination frequency	1.08	0.44	2.64	1.57	2.80	2.60	4.62	2.63	2.73	3.04	30.3	100
Recomb. freq. corrected*	0.92	1.02	1.47	1.53	2.66	2.68	2.78	3.58	4.28	4.57	18.2	100

* Corrections were carried out to make the phage titre 100.

tions. However, the reverting mutants *A-266*, *-267*, and *-229* were not overlapped by any of the deletions, though they showed recombination frequencies of 0.64, 0.71, and 0.76% respectively with *A-246*. This contradiction is probably due to the decrease in recombination frequencies in the crosses with the three deletion mutants.

In Table 3, the results obtained from the crosses between 11 *motB* mutants and *A-246* are presented. In these crosses, recombination frequencies ranged from 0.92 (*B-215*) to 18.2% (*B-211*), and these values were fairly high as compared with those shown in Table 2. It is not unexpected that the frequency in the cross between mutants in different functional units are higher than that with mutants within the same unit. Mutant *B-211* showed highest recombination frequency among *motB* mutants, and its site was inferred to be farthest from *motA*. *B-211* is the only mutant of *motB* which complements normally the majority of the weak-complementing *motA* mutants. This character is perhaps due to the location of *B-211* far from the sites of other *B* mutants.

In recombination tests between weak-complementing mutants and *A-246*, the tests between two normal complementing mutants (*A-259* and *B-295*) and *A-246* were carried out simultaneously to compare the recombination frequencies with those obtained from the other two series of tests (Tables 2, 3). The results are shown in Table 4. The mutational sites of the 12 weak-complementing *A* mutants were not apparently clustered in any particular region but dispersed randomly in the *motA* functional unit. *A-297* was found to be a deletion overlapping *A-230* and *A-234*. *A-297* and *A-234* were used for the recombination tests and showed the frequencies of 1.31 and 1.57%, respectively. A reverting mutant *A-263* which was not overlapped by *A-297* showed a recombination frequency of 1.56%; consequently its mutational site was assigned between *A-297* and *A-234*. This contradiction would be explained if *A-297* shows decreased recombination frequency on account of the deletion. *B-227*, which showed a very weak response to *motA* mutants in the complementation tests, was located nearest to *A-246*. *A-259* and *B-295* showed recombination frequencies of 0.42 and 2.91% in this series. These values correspond or are close to the values obtained from the other two series of tests (0.42 and 2.78). Therefore, the recombination frequencies obtained from the cross with the weak-complementing mutants can be compared with those shown in Tables 2 and 3. In Figure 3, the results of the three series of recombination tests are shown. The order of the mutational sites, especially of the adjacent sites, should be considered to be tentative, because the mapping depends on the recombination frequencies obtained from the crosses with *A-246* only. In order to map precisely, reciprocal transduction tests between other mutants are required. However, the approximate location of the mutational sites can be assigned as in Figure 3.

DISCUSSION

The results of the complementation tests showed that the sites of mutations leading to flagellar paralysis were grouped in three functional units: *motA*, *motB*, and *motC*. *motA* and *motB* adjoin each other and *motC* is located apart

TABLE 4
Recombination frequency in transduction from weak-complementing mutants to A-246

	<i>motA</i> Mutant No.										<i>motB</i> No.			
	300	258	259†	237	219	294‡	233	207	297‡	263	234	249	280	TM2
Phage titre (%)	94.5	139.0	94.4	236.2	70.8	236.2	166.8	152.8	95.8	225.2	77.8	166.8	137.5	
Recombination frequency	0.19	0.31	0.40	1.18	0.53	1.81	1.44	1.95	1.25	3.50	1.22	3.62	3.69	
Recomb. freq. corrected*	0.20	0.22	0.42	0.50	0.75	0.77	0.86	1.28	1.31	1.55	1.57	2.17	2.68	
												0.27	1.50	2.91
												0.36	1.98	4.85
												134.8	133.2	166.5
														100

* Corrections were carried out to make the phage titre 100.
† Deletion mutant.
‡ Normally complementing mutants included as controls.

FIGURE 3.—Genetic map of *motA* and *motB*. The results of three series of transduction tests are arranged as follows: crosses between *A-246* and *motA* mutants (top, right); between *A-246* and *motB* mutants (top, left); and between *A-246* and weak-complementing mutants (bottom). The scale indicates recombination frequencies (percent). The upper and lower maps are comparable with each other except in respect of close mutational sites (see text). *A-238*, -277, and -282 of the upper map (cf. Figure 2), and *A-294* and -297 of the lower map are deletions.

from them. *motC* is transduced jointly with *H1* at a high frequency. Whether *motC* is located on the *his* side or *try* side in relation to *H1*, and whether *motA* and *motB* are located on the same side as *motC* in relation to *H1* or the opposite side, remains to be investigated.

The two-factor transduction tests revealed that the representative mutational sites of the *motA* and *motB* could be arranged in a linear sequence. In *motB*, *B-227* was located closest to *motA* and *B-211* was farthest from it. *B-227* is a weak-complementing mutant showing little response to all the *motA* mutants tested. On the contrary, *B-211* complements normally the majority of the weak-complementing *motA* mutants. The behavior of these two mutants suggests that the closer the two mutational sites are, the weaker the complementation.

In order to map the mutational sites by means of two-factor transduction tests, generally all pairwise crosses are carried out and the frequencies of the wild-type recombinants are compared for each pair of mutants (HARTMAN 1956). In the present investigation, fortunately, the mutant *A-246* was found to be located near the boundary of the two functional units by virtue of the long deletion *B-292*, so the mapping has been carried out between *A-246* and *motA* or *motB* mutants. In order to avoid as many errors as possible, one series of crosses was performed by use of the same recipient culture simultaneously. However, inherent error will occur, such as the correction of the recombination frequency by the potency of transducing phage. Therefore, the accurate order of the mutational sites, especially those of the adjacent mutational sites, should be determined only after reciprocal transduction tests with other mutants.

The mutational sites of 14 weak-complementing mutants were not located in a specific region, but distributed throughout the whole functional unit. They show normal complementation in crosses with the wild-type and *motC* mutants. Therefore, the character of these mutants is not a result of cytoplasmic defect. These mutants were tentatively assigned to *motA* and *motB*. It is difficult to interpret these mutants by the present genetic data, because whether *motA* and *motB* control different protein molecules respectively or together control a single protein molecule, are unknown. For the latter the interpretation will be changed according to whether two different polypeptide subunits are elicited by

separate *motA* and *motB* units or one polypeptides is made along the entire *A* and *B* region to form a single active protein molecule. The character of these mutants is similar to that of the *Dab* mutants of the *his* operon (HARTMAN, HARTMAN, and ŠERMAN 1960; LOPER *et al.* 1964): they do not complement the mutants of either *a* or *b* functional unit of *hisD*.

A biochemical analysis of flagellar movement has not been carried out. It has been suggested that the bond energy of ATP is involved in motility (DE ROBERTIS and PELUFFO 1951; DE ROBERTIS and FRANCHI 1952; SHERRIS *et al.* 1957). However, the ATP content in the motile wild-type cell was not significantly different from that of the paralyzed cells (representatives of *motA* and *motB*) (ENOMOTO 1962; unpublished data). The energy required for flagellar propulsion is less than 0.1% of that of the total cell (HERBERT 1951), so that the difference in ATP content may not be detectable. From the finding that (1) ATP and ATPase activity are not detected in flagella detached from both wild-type and paralyzed cells (ENOMOTO 1962; unpublished data), and that (2) the flagellar protein, morphology, and the configuration by X-ray diffraction (BEIGHTON *et al.* 1958) are not different in the two genotypes, it is proposed that the defect leading to flagellar paralysis is located in the cytoplasm, probably in the basal granules. On the basis of these genetic data with the paralyzed mutants, biochemical investigations of flagellar movement are in progress.

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SUMMARY

Ninety-seven paralyzed mutants were divided into three complementation groups by abortive transduction tests: *motA* with 64; *motB* with 30; and *motC* with 3. *motA* and *motB* were found to constitute adjoining functional units because of the occurrence of joint transduction of *motA* and *motB*, and of a deletion mutation covering the whole *motB* and a part of *motA*. *motC* was transduced jointly with *H1*, a phase-1 flagellin gene, and the order of the three mutational sites within *motC* was determined. The order of the representative mutational sites in *motA* and *motB* was also determined tentatively by deletion mapping and two-factor transduction tests.

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