# The genetic basic of flagellar structure in Escherichia coli

by Aletha Markusen

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology
Montana State University


Abstract:
The genetic basis of the motility--non-motility difference between E. coli K 12 and E. coli E was examined by transduction and conjugation experiments. A single gene difference was revealed. Since B is non-flagellated, this gene appears to be analogous to the fla genes of Salmonella and to be concerned with the organization of flagella. In view of the single gene difference, either fla+ also renders flagella functional, or E. coli B already possesses genes (mot+) for flagellar function. Fla may be linked to lac (a gene for lactose fermentation).

The specificity of the flagellar antigen is not determined by the fla gene, since some motile recombinants do not cross-react appreciably with antiserum against the motile parent. One gene for antigenic specificity, (ant), may be linked to mtl (a gene for mannitol fermentation).

At least one recombinant, obtained by conjugation, is of an unprecedented type, possessing flagellar antigens but no flagella.

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ALETHA MARKUSEN

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Approved:

Louis D. Smith
Head, Major Department

P. D. Skaar
Chairman, Examining Committee

Leon D. Johnson
Dean, Graduate Division

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## ABSTRACT

The genetic basis of the motility--non-motility difference between *E. coli* K 12 and *E. coli* B was examined by transduction and conjugation experiments. A single gene difference was revealed. Since B is non-flagellated, this gene appears to be analogous to the *fla* genes of *Salmonella* and to be concerned with the organization of flagella. In view of the single gene difference, either $fla^+$ also renders flagella functional, or *E. coli* B already possesses genes ($mot^+$) for flagellar function. *Fla* may be linked to *lac* (a gene for lactose fermentation).

The specificity of the flagellar antigen is not determined by the *fla* gene, since some motile recombinants do not cross-react appreciably with antiserum against the motile parent. One gene for antigenic specificity, (*ant*), may be linked to *mtl* (a gene for mannitol fermentation).

At least one recombinant, obtained by conjugation, is of an unprecedented type, possessing flagellar antigens but no flagella.

## INTRODUCTION

In Salmonella, it has been shown that there are several genetic factors which control the structure and function of flagella. Stocker and co-workers (1953) found that non-flagellated (0) Salmonella cells could be converted to cells with functional flagella by transduction. Further, they found that the H antigens of the transductants were characteristic of the flagellated strains from which the non-flagellated strains had been derived. This was interpreted to mean that genes responsible for antigenic specificity are retained in the non-flagellated strains, that is, that the mutation causing lack of flagella does not affect these genes. When the flagellar gene is supplied in transduction, the inherent antigenicity of the non-flagellated strain is expressed.

When non-flagellated strains were treated with phage lysates produced on certain other non-flagellated strains, motile forms were obtained showing that absence of flagella in the two strains was due to mutations of non-homologous genes. By employing various combinations of non-flagellated strains (Stocker et al.) found that several genetic factors were involved, mutation of any one of which might result in lack of flagella.

Phage lysates produced on non-motile flagellated (paralyzed) forms conferred motility upon non-flagellated strains indicating that genes for flagellar presence and for flagellar function are separable.

Each of two paralyzed strains also produced swarms on motility medium when treated with lysates of the other, indicating that at least two separate genetic factors are involved in flagellar function.

Some motile transductants were slow swarmers. A higher rate of swarming could be brought about by a subsequent transduction with a lysate produced on rapidly swarming strains.

Flagellated Salmonella are either monophasic or diphasic. The experiments of Lederberg and Edwards (1953) show that in diphasic strains, there are genetically separable genes determing antigens characteristic of each phase. The experiments of Lederberg and Iino (1956) provide information on the mechanism by which only one of these genes is expressed in a given cell.

Close linkage between one antigen gene and one gene for presence of flagella has been demonstrated by the joint transduction of the two factors (Stocker et al.).

The general picture which emerges is one of several fla genes (determining presence of flagella), at least two mot genes (determining function of flagella), at least one gene determining speed of swarming, and at least two ant genes (determining antigenic specificity). These genes appear to be scattered through the bacterial genome.

Furness (1958), in studies of a cross between E. coli K 12 (F+ motile) and E. coli B (F- non-motile) has reported that:

1. Motile recombinants are obtainable.
2. The non-motility of B is due to a single genetic factor located in the vicinity of a gene for tyrosine synthesis.
3. The motile recombinants are antigenically like the K 12 parent in spite of the fact that all recombinants are

like B for most of the other known markers.

In short, these results suggest something quite different for E. coli than for Salmonella. Fla, mot, and ant activity would all appear to be the pleiotropic concern of one gene, or of a cluster of closely linked genes.

There are several deficiencies in Furness' analysis. First, he does not appear to have considered the complicating factors which are introduced by the unstable heterogenotes which usually issue from a K 12 x B cross. (Bryson et al., 1955). Second, his results provide little assurance that the motile recombinants might not all have involved rather extensive transfer of the K 12 genome. Third, he does not report a search for "paralyzed" recombinants. Occurrence of such forms would point to separable fla and mot genes. Fourth, his statement that the recombinants were antigenically identical to the K 12 parent was not reinforced by any evidence that they possessed full capacity for absorption.

The purpose of the present investigation was to attempt to verify Furness' results and to determine whether the genes for flagellar characters, in contrast to most other genetic factors (Demerec, 1959), are quite differently disposed in the E. coli genome than in that of Salmonella.

# MATERIALS AND METHODS

## Bacterial Strains

K 12 strains:

MSC18 is CS2 made F+ by growth with CS11. CS2 is a highly motile F- proline-requiring derivative of 58-161 which, in turn, is deficient for methionine and ferments lactose, galactose, arabinose, xylose, mannitol, and maltose.

CS11 is a streptomycin-resistant F+ derivative of W1177 which is motile but not highly motile, deficient for thiamine, threonine, and leucine. It ferments maltose but not lactose, galactose, arabinose, xylose, or mannitol.

B strains:

IMN64 is a streptomycin-sensitive shikimic acid auxotroph of B/r. It is non-motile and non-flagellated and ferments lactose, galactose, arabinose, xylose, and mannitol but not maltose.

IMN60 is a streptomycin-sensitive, $T_1$ resistant, tryptophan auxotroph of B/r. It is non-motile and non-flagellated and ferments lactose, galactose, arabinose, xylose, and mannitol but not maltose.

## Bacteriophage Strains

The phage P1kc of Lennox (1955) was used in transduction experiments.

## Culture Media

Stock cultures were routinely maintained on Difco nutrient agar.

All culture media were made with distilled water and weights cited are

designed for one liter volumes.

Defined medium used in selection of prototrophs was Davis Minimal Medium (DMA) (Lederberg, 1950) which contains: Glucose, 1 g; $K_2HPO_4$, 7 g; $KH_2PO_4$, 2 g; $Na_3$citrate$\cdot 5\ H_2O$, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1 g; agar, 15 g. Glucose and agar were sterilized together in 500 ml of distilled water and mixed with the other constituents after sterilization.

The semi-solid agar used to detect motility is that of Edwards (1942). It contains: Bacto-Peptone, 10 g; yeast extract, 3 g; agar, 4 g; gelatin, 80 g; sodium chloride, 5 g. Gelatin was sterilized separately in 500 ml of distilled water and added to the other constituents after sterilization. pH was adjusted to 7.0 with sterile 1 N NaOH after mixing the two components.

Eosin methylene blue medium was prepared according to the formula of Lederberg (1950). It contains: Casein digest (N Z Case), 10 g; yeast extract, 1 g; sodium chloride, 5 g; $K_2HPO_4$, 2 g; eosin Y, 0.4 g; methylene blue, 0.065 g; agar, 15 g. Appropriate sugars were added to give a final concentration of 1%.

L broth was prepared according to the formula of Lennox (1955). It contains: Tryptone, 10 g; yeast extract, 5 g; sodium chloride, 5 g; glucose, 1 g. The pH was adjusted to 7.0 with 1 N NaOH.

L agar was prepared by adding 10 g of agar to a liter of L broth. Sterile $CaCl_2$ was added to the agar before pouring to give a final concentration of $2.5 \times 10^{-3}$ M. The top layer (soft) agar used in preparation of lysate was prepared by combining: Bacto nutrient broth powder, 8 g; sodium chloride, 5 g; and agar, 6.5 g.

Phage diluting fluid contains: Sodium chloride, 3 g; Bacto-Peptone, 1 g; $MgSO_4$, 0.12 g.

Cultures used as antigen in serological tests were grown in Difco nutrient broth.

Difco Pennassay broth was used to grow cultures used in conjugation experiments.

Brom cresol purple broth contains: Beef extract, 3 g; peptone, 5 g. pH was adjusted to 7.0 with 1 N NaOH and a solution of brom cresol purple was added to give a final concentration of 0.004%. Lactose or maltose was incorporated into the broth before sterilizing to give a final concentration of 1%.

Streptomycin agar was prepared by spreading 0.1 ml of an aqueous solution containing 0.02 mgm on the surface of a nutrient agar plate.

When amino acid or vitamin supplements were added to minimal agar, a solution containing 0.2 mgm was spread on a plate of medium.

Stains

Leifson's Modified Flagella Stain (B-B-L) was used in flagella staining. One gram of the dry powder was dissolved in a mixture of 33 ml of distilled water and 16 ml of 95% ethyl alcohol.

For flagella staining, new slides were washed in a non-soap-containing detergent, rised thoroughly with warm tap water, rinsed again with distilled water, and allowed to air-dry. Suspensions of 24 hour nutrient agar slant cultures were made in 1 ml of distilled water and carefully spread on the slide with a wire loop. Slides were allowed to dry and then were flooded

with the freshly filtered stain. The stain was allowed to stand for ten minutes and then was washed off with tap water. Slides were blotted dry and examined under oil immersion.

Preparation of Lysates

Phage lysates were prepared according to the general method of Swanstrom and Adams (1951). Two drops of an overnight culture of the donor strain of bacteria and 1 ml of P1kc which had been found to have a titer of $1.7 \times 10^{10}$ on E. coli strain K 12 were added to 2 ml of molten soft agar at 45 C. $CaCl_2$ was added to give a final concentration of $2.5 \times 10^{-3}$ M in order to allow phage adsorption.

After it was mixed, the soft agar was poured over L plates which had been prepared the day before and allowed to remain at room temperature. The soft agar was allowed to harden and the plates were incubated at 37 C in an upright position.

After seven hours of incubation, 5 ml of L broth plus $CaCl_2$ were added to the soft agar layer and the plates were incubated for three hours, after which the fluid was removed aseptically with a pipette, and centrifuged in sterile centrifuge tubes to sediment the cells. The supernate was decanted and $CHCl_3$ was added to give a final concentration of 0.1 ml per 5 ml of lysate. $CHCl_3$ was allowed to evaporate by pouring the lysate into sterile open Petri plates and rotating gently for a few minutes. The lysate was stored at 5 C in sterile screw-cap tubes.

Phage Assays

Phage assays were carried out according to the agar layer method of Gratia (Adams, 1959). Serial dilutions of the lysate up to 1 x $10^{-5}$ were made in phage diluting fluid. Five ml cultures of the sensitive bacterial strains were grown for three hours at 37 C in L broth. Two drops of the culture were added to 2 ml of soft agar at 45 C. One ml of lysate plus $CaCl_2$ was added and the mixture poured onto the surface of L plates. Plates were incubated upright at 37 C and later examined for plaques.

Transduction Procedures

The transductions were carried out with a 10 ml Pennassay broth culture of IMN60 which had been incubated for three hours at 37 C to give a final concentration of approximately 1 x $10^8$ cells per ml. The cultures were divided into two 4 ml volumes and to one of the cultures was added 0.4 ml of P1kc grown on MSC18 which had been found to have a titer of 2.9 x $10^7$ on IMN64. Two drops of $CaCl_2$ solution were also added to this culture. Both cultures were further incubated for a half hour at 37 C. They were then centrifuged and the pellet resuspended in 1 ml of saline. One-tenth ml of each suspension was plated on a separate DMA plate and an equal volume of each suspension was spotted on a plate of semi-solid motility medium. A second plate of motility medium was spotted with 0.1 ml of the lysate to serve as a control against the possibility of the presence of motile MSC18 or other contaminating motile forms in the lysate. A third plate spotted with the IMN60 cell suspension served as a control to detect the presence

of motile mutants in the IMN60 cell suspension. The entire procedure was also carried out with a culture of MSC18 in order to check for the presence of contaminating prototrophs in the lysate. All plates were incubated at 37 C.

To test for prototrophy, cultures were incubated overnight at 37 C in Pennassay broth, centrifuged, washed twice in normal saline and resupended in 2 ml of saline. One-tenth ml quantities of the suspensions spread on DMA or on DMA with appropriate supplement were incubated at 37 C.

Conjugation Procedures

Crosses between the K 12 strain, CS11 and the B strain, IMN64, and between CS11 and the B strain, IMN60 were carried out according to the methods of Lederberg et al. (1951). Each of three 5 ml aliquots of Pennassay broth were inoculated with one of the cultures and incubated for 16 hours at 37 C. Cultures were then centrifuged, washed twice in sterile saline and resuspended in 1 ml of saline. One-tenth ml portions of the suspensions of each culture were spread on plates of DMA to check for the appearance of prototrophic mutants as well as for the presence of contaminants in the cultures. Other plates were inoculated with 0.1 ml portions of CS11 plus a 0.1 ml portion of one of the B strains. Two plates were inoculated with each combination. The plates were inverted and incubated at 37 C for 48 hours.

Inoculum from prototrophic recombinants which arose on DMA plates was streaked on plates of nutrient agar to facilitate segregation of mating

pairs. After 24 hours of incubation at 37 C, discrete colonies were picked and plates of DMA inoculated to check for prototrophy. Growth from single colonies appearing on DMA were transferred to agar slants, which were incubated overnight at 37 C and stored at 5 C for future use.

Preparation of Vaccine

Kolle flasks of nutrient agar were inoculated with nutrient broth cultures of MSC18 and incubated overnight at 37 C. Growth was removed carefully by washing with sterile saline containing 0.2% formalin. Nutrient agar plates were streaked with the cell suspension and incubated at 37 C to test for viability of cells in the formalin treated culture. Tubes of brain heart infusion agar were inoculated and incubated at 37 C to check for the presence of viable anaerobic contaminants. No growth was observed on either plates or stabs after seven days of incubation.

The cells were shaken by hand in screw-cap test tubes containing glass beads in order to obtain a homogeneous suspension which was filtered through sterile gauze and sterile glass wool and standardized with a McFarland's nephelometer to a density of approximately $1 \times 10^9$ cells per ml.

Production of Antiserum

Fifty ml of blood was drawn from the marginal vein of the ear of a rabbit. The blood was centrifuged and the serum decanted and stored in the deep freeze for future use as a control in agglutination tests.

Vaccine was administered intravenously to the rabbit according to the following schedule: (Carpenter, 1956)

first day----------0.1 ml

fourth day----------0.3 ml

eighth day----------0.5 ml

eleventh day----------1.0 ml

fifteenth day----------2.0 ml

Three more 1.0 ml injections were given at weekly intervals and the rabbit was bled on the fifty-second day.

The blood was centrifuged, the serum decanted and frozen for future use in serologic tests. When the antiserum and control serum were subsequently thawed for use in agglutination tests, an aqueous solution of merthiolate was added to give a final concentration of 0.02%.

Preparation of Absorbing Antigen

Kolle flasks of nutrient agar were inoculated with broth suspensions and incubated at 37 C for 48 hours. The growth was washed off with L broth containing a final concentration of 0.5% formalin. Each flask was washed with 4 ml of broth in order to provide a fairly uniform density of the suspension.

Preparation of Test Antigens for Absorption Tests

Flasks containing 250 ml of L broth which had been inoculated with the test organisms were incubated overnight at 37 C. Formalin was added to give a final concentration of 0.5%. Suspensions used in testing for O antigens were heated to 120 C for two hours in the autoclave.

Serum Absorption Procedure

Four ml of the cell suspensions were centrifuged, decanted and 2.0 ml of a 1:5 dilution of antiserum added to the packed cells. Cells and serum were incubated for one hour in a 56 C water bath, centrifuged to sediment the cells, and the serum decanted for use in agglutination tests. Absorptions were repeated as many times as necessary to deplete serum of antibody.

Agglutination Tests

Antiserum and control serum were diluted with saline to appropriate concentrations. Each tube contained 0.5 ml of diluted serum and an equal volume of test antigen and were incubated in a water bath at 56 C for two hours. Five-tenths ml portions of living broth cultures were used as antigen in tests listed in Tables II and III.

# RESULTS

## Transduction Experiments

Experiments were carried out to determine whether any genetic character could be transferred from the K 12 strain MSC18 to the B strain IMN60 by the usual transduction methods and, if so, whether motility could also be transferred in this manner.

IMN60 treated with P1kc phage grown on MSC18 was plated on DMA. After 48 hours of incubation at 37 C, 60 colonies were counted. No growth was observed on control plates of DMA which had been inoculated with IMN60 alone. This indicated that prototrophy had been acquired by transduction--not by mutation.

Samples of the same IMN60 culture treated with P1kc phage grown on MSC18 were plated on semi-solid medium. After 24 hours of incubation at 37 C, the motility plates were examined for swarms. Several small swarms were present surrounding colonies growing on the plates which had been inoculated with the phage-bacteria suspension. No swarms were observed on the plates on which growth had been produced from IMN60 alone and no growth was observed on the plates inoculated with the lysate only. This was interpreted to mean that the swarms produced were the result of transduction rather than of mutation. Furthermore, mutation to motility in no B strain ever has been reported (Furness, 1958). Contamination by motile contaminants was ruled out by sterility of the plate inoculated with lysate alone.

Cultures obtained from these swarms, when tested for tryptophan

deficiency and for ability to ferment maltose, were found to exhibit the reactions of IMN60. (They were also found to ferment lactose, galactose, arabinose, and xylose. In IMN60 and MSC18, these markers are alike). One of these transductants was studied further and will be referred to as MSC39 throughout the paper.

Evidence obtained from this transduction experiment suggests that, in E. coli strain B as in non-flagellated Salmonella species, motility can be conferred upon a non-flagellated strain by the introduction of a single gene. This would be a fla gene according to the terminology of Salmonella genes. A corollary of this inference is that, if the array of flagella genes in E. coli is like that in Salmonella, strain B is already $mot^+$.

MSC39 was studied serologically to determine whether or not ant genes were closely linked to the fla genes. Results of the studies are summarized in Table I.

When MSC39 was tested with antiserum prepared against MSC18, strong agglutination was observed only at low (1:400) dilutions of antiserum whereas MSC18 was agglutinated by a 1:6400 dilution. Neither gave agglutination with a 1:200 dilution of control non-immune serum. Thus, MSC39 appears to be distantly related serologically to MSC18. The low titer agglutination of MSC39 by antiserum cannot be explained on the basis of O antigen since agglutination tests using heated antigen gave negative results.

It will be noted (Table I) that CS11 and MSC18 reciprocally remove antibody from anti-MSC18 serum and can be used interchangeably as antigen.

According to Ørskov and Ørskov (1960) possession of K antigen by K 12

Table I

Results of serological tests involving MSC39

| Antigen | MSC18 Antiserum | | | | | | | | | Control Serum |
|---|---|---|---|---|---|---|---|---|---|---|
| | Dilutions of Serum | | | | | | | | | |
| | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:12800 | 1:25600 | 1:51200 | 1:200 |
| Unheated | | | | | | | | | | |
| MSC39 | + | + | - | - | - | - | - | - | - | - |
| MSC18 | + | + | + | + | + | + | + | - | - | - |
| CS11 | + | + | + | + | + | + | + | + | - | - |
| Heated | | | | | | | | | | |
| MSC39 | - | - | - | - | - | - | - | - | - | - |
| CS11 | - | - | - | - | - | - | - | - | - | - |
| Antiserum Absorbed with Heated MSC39 Cells | | | | | | | | | | |
| Unheated | | | | | | | | | | |
| MSC39 | + | + | + | + | - | - | - | - | - | |
| CS11 | + | + | + | + | + | + | + | + | - | |
| Antiserum Absorbed with Unheated MSC39 Cells | | | | | | | | | | |
| Unheated | | | | | | | | | | |
| MSC39 | - | - | - | - | - | - | - | - | - | |
| CS11 | + | + | + | + | + | + | + | + | - | |
| MSC18 | + | + | + | + | + | + | + | + | - | |
| Antiserum Absorbed with Unheated CS11 Cells | | | | | | | | | | |
| Unheated | | | | | | | | | | |
| CS11 | - | - | - | - | - | - | - | - | - | |
| MSC18 | - | - | - | - | - | - | - | - | - | |
| Antiserum Absorbed with Unheated MSC18 Cells | | | | | | | | | | |
| Unheated | | | | | | | | | | |
| CS11 | - | - | - | - | - | - | - | - | - | |
| MSC18 | - | - | - | - | - | - | - | - | - | |

Heated = 2 hours at 120 C, + = Agglutination, - = No agglutination

strains of E. coli is doubtful. However, in the absence of experimental evidence to the contrary, presence of a heat labile K antigen on MSC39 cannot be excluded as a possibility. To test the similarity of MSC39 and MSC18 further anti-MSC18 serum absorbed with MSC39 was tested for its ability to agglutinate MSC18. It was found that the titer of antiserum at which MSC18 agglutinated was not decreased by absorption with MSC39. These results could mean that MSC39 and MSC18 do not have a common H antigen but do share some other heat labile (possibly K) antigen. Removal of K antibody or minority H antibody by absorption with MSC39 would not be expected to deplete, detectably, the overall anti-MSC18 serum. An alternate possibility is that there are antibodies in anti-MSC18 serum against two H components, and that MSC39 possesses only the minority antigen.

In any case, an important conclusion can be drawn from these results: Determination of antigenic specificity and flagellar presence is not the concern of one gene but of two. This contrasts with Furness' conclusions and agrees with the situation known to exist in Salmonella.

Conjugation Experiments

Crosses were made between CS11 and IMN60 and between CS11 and IMN64 in an attempt to locate the fla gene and, possibly, also the ant genes. Selection was made for prototrophy. Approximately 100 colonies were counted on each plate on which a cross had been made. No colonies appeared on plates which had been inoculated with a single culture, thus ruling out the presence of prototrophic mutants. Fifty colonies were picked from each cross and,

after purification on nutrient agar and verification of prototrophy, were stored at 5 C on agar slants for use in further studies.

Before storage, a test was made for ability to produce swarms on semi-solid medium. Broth suspensions were prepared from the agar slant cultures and were used to inoculate plates of semi-solid medium. No swarms appeared after twenty-four hours of incubation at 37 C. This does not necessarily mean that no recombinants were fla$^+$ since fla$^+$ K 12 strains often fail to swarm on first passage on semi-solid medium.

Further tests on CS11 x IMN60 recombinants were deferred. Nutrient broth suspensions from the CS11 x IMN64 recombinants were used to inoculate media for serological and biochemical tests. Results of these tests are contained in Table II. Since B strain are F- or receptor cells, recombinants generally are like the B parent genetically except for those genes which are closely linked to the selective marker (shi in the case of IMN64, try in the case of IMN60). The high number of streptomycin-resistant and of xyl$^-$ and mtl$^-$ colonies therefore is in agreement with the inference that shi is closely linked to these genes (Bryson et al., 1955).

The occurrence of many recombinant cultures within which cells varied with respect to fermentative markers suggests that these cells were or had been diploid or aneuploid. Segregation in heterozygous bacterial cells was first reported by Lederberg (1951). A cross between two auxotrophic K 12 strains, one of which was lac$^+$, the other lac$^-$, produced uniformly lac$^+$ colonies on synthetic medium. When single colonies were picked and streaked again on EMB lactose medium, both lac$^-$ and lac$^+$ forms appeard. Subsequent

Table II

Results of serological and biochemical tests performed on freshly isolated recombinants obtained in a cross between E. coli CS11 $S^R$ motile F+ ($T^-$, $L^-$, $B_1^-$, $lac^-$, $gal^-$, $arab^-$, $xyl^-$, $mtl^-$) and E. coli IMN64 $S^S$ non-motile F- ($try^-$, $lac^+$, $gal^+$, $arab^+$, $xyl^+$, $mtl^+$)

| | | *First Broth Culture | | | | | | *Second Broth Culture | *Third Broth Culture | After Five Passages on Semi-solid Agar | | | After Eight Passages on Semi-solid Agar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | lac | gal | arab | xyl | mtl | Anti-serum | Anti-serum | Anti-serum | Anti-serum | Control serum | Swarm | Anti-serum | Swarm |
| 1 | R | + | + | + | - | - | - | - | - | - | - | - | - | - |
| 2 | S | + | + | + | - | - | - | - | - | - | - | - | - | - |
| 3 | R | + | m | m | + | - | - | - | - | - | - | - | - | - |
| 4 | S | + | m | + | - | + | A | A | A | A | - | - | A | - |
| 5 | S | + | + | + | + | m | - | - | - | - | - | - | - | - |
| 6 | R | + | m | + | + | + | - | - | - | - | - | - | - | - |
| 7 | R | + | m | 0 | + | + | - | - | - | - | - | - | - | - |
| 8 | R | + | m | 0 | - | + | - | - | - | - | - | - | - | - |
| 9 | R | + | + | m | + | + | - | - | - | P | - | - | P | - |
| 10 | R | + | + | + | + | m | - | - | - | - | - | - | - | - |
| 11 | R | + | + | m | + | + | - | - | - | - | - | - | - | - |
| 12 | R | + | + | + | + | + | - | - | - | P | - | - | P | - |
| 13 | S | + | m | + | + | + | - | - | - | - | - | - | - | - |
| 14 | R | + | m | + | - | - | - | - | - | - | - | - | - | - |
| 15 | R | + | m | + | + | m | - | P | P | - | - | - | - | - |
| 16 | R | + | m | + | + | m | - | P | - | - | - | - | - | - |
| 17 | R | + | + | + | - | - | - | P | A | A | - | - | A | Sw |
| 18 | R | + | m | m | + | m | - | - | - | - | - | - | - | - |
| 19 | R | + | m | + | - | - | - | - | - | - | - | - | - | - |
| 20 | R | + | + | + | - | - | - | P | A | - | - | - | - | - |
| 21 | S | + | m | + | - | - | - | - | - | - | - | - | - | - |
| 22 | R | + | m | + | - | - | - | P | P | P | - | - | P | - |
| 23 | R | + | m | m | - | - | - | P | - | - | - | - | - | - |
| 24 | S | + | + | + | + | - | - | - | - | - | - | - | - | - |
| 25 | S | - | m | + | - | - | - | P | - | - | - | - | - | - |
| 26 | S | + | + | m | m | m | - | - | - | - | - | - | - | - |
| 27 | R | + | m | + | + | - | - | - | - | P | - | - | P | - |
| 28 | R | + | m | + | + | + | - | - | - | - | - | - | - | - |
| 29 | R | + | m | + | m | + | - | - | - | - | - | - | - | - |
| 30 | R | + | m | + | m | + | - | - | - | - | - | - | - | - |
| 31 | R | + | m | + | + | + | - | - | - | - | - | - | - | - |
| 32 | R | + | + | + | + | + | - | - | - | - | - | - | - | - |
| 33 | S | + | + | + | + | + | - | - | - | - | - | - | - | - |
| 34 | R | + | + | + | + | + | - | - | - | A | - | Sw | A | Sw |
| 35 | R | + | + | + | + | + | - | - | - | - | - | - | - | - |
| 36 | R | + | + | + | + | m | - | - | - | - | - | - | - | - |
| 37 | R | + | + | + | + | + | - | - | - | - | - | - | - | - |
| 38 | R | + | + | + | + | m | - | P | A | - | - | - | - | - |
| 39 | R | + | + | + | - | m | - | - | - | A | - | Sw | A | Sw |
| 40 | R | + | m | m | m | m | - | - | - | A | - | Sw | A | Sw |
| 41 | S | + | m | m | m | + | - | - | - | - | - | - | - | - |
| 42 | R | + | m | m | + | + | - | - | - | - | - | - | - | - |
| 43 | S | + | m | m | m | + | - | - | - | - | - | - | - | - |
| 44 | S | + | m | m | m | + | - | - | - | - | - | - | - | - |
| 45 | S | + | + | + | - | + | - | - | - | - | - | - | - | - |
| 46 | R | + | m | m | + | - | - | P | P | P | - | - | P | - |
| 47 | S | + | m | m | + | + | - | - | P | - | - | - | P | - |
| 48 | R | + | + | + | - | - | - | - | - | P | - | - | - | - |
| 49 | R | + | m | m | - | + | - | - | - | - | - | - | - | - |
| 50 | S | + | + | + | + | - | A | A | A | - | - | - | - | - |
| CS11 | R | - | - | - | - | - | A | A | A | | | | | |
| IMN64 | S | + | + | + | + | + | - | - | - | | | | | |

0 = no growth after two trials, + = fermentation, - = no agglutination or no visible fermentation, m = mixed (fermentative and non-fermentative), R= streptomycin resistance, S = streptomycin sensitivity, A = strong agglutination in 1:1000 serum, P = partial agglutination in 1:100 serum, Sw = swarming.

* First, second, and third broth cultures were obtained by successive transfer.