Genetic studies on a heat stable surface antigen of Escherichia coli K12
by Aletha Sylvia Markusen

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
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Occasional mutations from HSA+ to HSA- and the reverse have occurred simultaneously with mutations of other genes not obviously related physiologically.

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ABSTRACT

The flocculent agglutination produced by many strains of *E. coli* K12 in response to antiserum is due in part to the presence of flagellar antigen and in part to a heat stable antigen (HSA) not associated with flagella.

Presence of HSA is not obviously correlated with F status. Appropriate crosses show its genetic basis not to be closely linked to the TLB_1 region.

Occasional mutations from HSA^+ to HSA^- and the reverse have occurred simultaneously with mutations of other genes not obviously related physiologically.

INTRODUCTION

In an earlier investigation (Markusen, 1960), an E. coli hybrid between strain K12 (motile) and strain B (non-motile) was found to be non-motile even after repeated passages on motility medium. Upon examination by light and electron microscopy, it was found also to be non-flagellated. Nonetheless, cultures of this hybrid strain (MSC56) were agglutinated by high dilutions of antiserum which had been produced in response to its K12 parent. The aggregate formed in the agglutination resembled flagellar agglutination both in appearance and in the kinetics of its formation.

Close serological similarity between functionless molecules and their normal homologues has been demonstrated in other studies, for example the similarity between a cross-reacting protein (CRM) and tryptophan synthetase (Yanofsky, 1960). Furthermore in Salmonella the genetic loci determining flagellar antigen specificity (the H loci) are separable from the genes for flagellar structure (Stocker et al, 1953). Therefore, it was postulated that MSC56 was an hitherto undescribed recombinant type which carried (and expressed) the flagellar antigen specificity genes of K12 but lacked the genes for flagellar structure.

The preceding working hypothesis has been examined and found to be unlikely. Instead, the antigen carried by MSC56 appears to be not of the classical "H" type, but rather an antigen with novel properties.

The finding that at least one K12 mutant lacks this antigen permits investigation of its genetic basis.

MATERIALS AND METHODS

A. Bacterial and Bacteriophage Strains

All bacterial strains used in the investigation are described in Table I. Wild type forms of both the virulent phage T5 and the temperate phage lambda (Adams, 1959) were used.

B. Culture Media and Supplements

All final concentrations given below are in grams per liter of distilled water except where indicated. All culture media were sterilized in the autoclave at 15 pounds pressure for 20 minutes.

Nutrient Agar: Difco nutrient broth 8, NaCl 5, agar 15.

Motility Medium: Bacto Casitone 10, yeast extract 3, agar 4, Difco gelatin 80, NaCl 5. The pH was adjusted to 7.0 after sterilization.

Pennassay Broth (Bacto Antibiotic Medium 3): Bacto-Beef Extract 1.5, Bacto-Yeast Extract 1.5, Bacto-Peptone 5.0, Bacto Dextrose 1.0, NaCl 3.5, K_2HPO_4 3.6, KH_2PO_4 1.3.

Eosin Methylene Blue Medium (EMB): Casein digest 10.0, yeast extract 1.0, NaCl 5.0, K_2HPO_4 2.0, Eosin Y 0.4, Methylene Blue 0.065, agar 15.0, sugar 10.0.

Davis Minimal Agar (DMA): K_2HPO_4 7.0, KH_2PO_4 2.0, crystalline sodium citrate 0.5, crystalline $MgSO_4$ 0.1, crystalline $(NH_4)_2SO_4$ 1.0, glucose 1.0, and agar 15.0. The salts were added to 500 ml of water and sterilized. The glucose and agar were sterilized together in 500

Table I. Bacterial Strains.

Strain designation	Source	New genetic markers
W677	E. coli K12**	T^-, L^-, B_1^-, lac^-, mal^-, xyl^-, mtl^-, V_1^r
W1177	W677	S^r
CS11	W1177	F+ (Source of F: W6)
MSC503	W677	mal^+
MSC501	W1177	mal^+, mtl^+, B_1^+
W1603	W1177	Lp_1^-, T^+
W2736	W1603	Lp_2^s
MSC505	MSC501	mtl^-
CS19	E. coli K12**	pa^-, mut^+
W6 (58-161)	E. coli K12**	M^-
MSC69	W6	F-
W1895	W6	Hfr Cavalli
CS101	W1895	V_1^r
MSC70	MSC69	F+ (Source of F: CS11)
MSC18	W6	Pro^-
IMN60	E. coli B*	try^-, V_{1a}^r
MSC39	IMN60	made mot^+, fla^+ by transduction with P1 grown on MSC18
IMN64	E. coli B*	shi^-, V_1^r
MSC56	CS11 x IMN64	prototrophic, lac^+, xyl^-, mtl^-, fla^-, mot^-, S^s
MSC500	MSC56	S^r

*E. coli B is mot^-, fla^-, lac^+, mal^-, xyl^+, mtl^+, S^s, V_{1a}^s, V_1^s, prototrophic.

**E. coli K12 is mot^+, fla^+, lac^+, mal^+, xyl^+, mtl^+, S^s, V_1^s, Lp_1^+, Lp_2^s, mut^-, V_{1a}^s, prototrophic.

Notations for genetic markers are as follows: T, threonine; L, leucine; B_1, thiamine; pa, phenylalanine; try, tryptophane; shi, shikimic acid; M, methionine; A, arginine; lac, lactose; mal, maltose; xyl, xylose; mtl, mannitol; S, streptomycin; V_1, sensitivity to T1 and T5; V_{1a}, sensitivity to T1 not T5; Lp_1, lysogenicity for lambda; Lp_2, ability to adsorb lambda; mot, motility; fla, presence of flagella; mut, mutability.

ml of water and added to the salt solution after sterilization (Lederberg, 1950).

Saline: NaCl 8.6.

Streptomycin: Dihydrostreptomycin sulfate was used in all tests for streptomycin resistance. All streptomycin broth and agar media were prepared to give a final concentration of 250 µg/ml.

Growth Factors: The following supplements were added to minimal agar plates wherever appropriate to give these final concentrations: Thiamine .0001, p-aminobenzoic acid .001, arginine .02, methionine .02, leucine .02, phenylalanine .02, tyrosine .02, tryptophane .02, threonine .04, proline .02.

C. Agglutination Procedures

1. Production of Antiserum: Fifty ml of blood were 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.

2. Preparation of Vaccine: Kolle flasks of nutrient agar were inoculated with nutrient broth cultures of appropriate strains and incubated overnight at 37 C. Growth was removed carefully by washing with sterile saline containing 0.2 per cent 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 also were inoculated and incubated at 37 C to check for the presence of viable anaerobic contaminants. No growth ever was observed on either plates or stabs after seven days incubation.

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

3. Preparation of Absorbing Antigen: Nutrient agar plates were inoculated with 0.1 ml quantities of an overnight Pennassay broth culture. The nutrient agar plates were incubated twenty-four hours at 37 C and the growth was removed by washing with 5.0 ml of formalinized (0.5%) saline.

4. Serum Absorption: A modification of the methods recommended by the Mackie and McCartney Handbook (Cruickshank, 1960) was followed. The formalinized cell suspensions were centrifuged and resuspended in successive volumes of the saline suspension several times in order to

obtain a suitable cell density. The concentrated suspension was added to an equal volume of a 1:5 dilution of complete antiserum and the mixture was incubated at 55 C in the water bath for twenty hours. The cells were again centrifuged and the supernatant fluid was tested for presence of homologous antibody by titration with Pennassay broth cultures. If the serum was found to be incompletely absorbed, it was reincubated with packed cells in order to remove remaining antibody without further diluting the antiserum. When the antiserum was found to be completely absorbed, it was filtered through a Seitz filter.

5. Agglutination Tests: For quantitative tests, two-fold serial dilutions of antiserum were made with formalinized saline. Aliquots of 0.5 ml of twenty-four hour Pennassay broth cultures were added to 0.5 ml of the serum dilution in each tube. All tests were incubated in the water bath at 55 C for two hours and read macroscopically by oblique light on a Kahn viewer.

6. Preparation of Antigens for Heat Stability Tests: Pennassay broth cultures were heated for the appropriate length of time in the water bath when temperatures below 100 C were indicated. For temperatures of 100 C and higher, cultures were heated in the autoclave.

D. Procedures Involving Crosses

1. F Infection Procedures: See section D, part 2 of Results.

2. Cross Procedures: Five ml quantities of sixteen hour Pennassay broth cultures were centrifuged, washed in saline, and resuspended in 1.0 ml of saline. The saline suspensions were mixed well

and 0.1 ml of the suspension of each culture involved in the cross was pipetted to a plate of DMA. The two suspensions were mixed well with a glass spreader and allowed to dry. Controls consisted of 0.1 ml quantities of each culture plated separately on DMA plates. All cultures were incubated for forty-eight hours and scored for prototrophic recombinants.

3. Scoring of Recombinant Markers: To score for prototrophy, an inoculum from well-purified recombinants was grown in broth and streaked to plates of DMA. To score for fermentative markers, similar broth cultures were streaked on appropriate plates of EMB. Streptomycin resistance was tested by streaking on nutrient agar plates containing streptomycin. Sensitivity to bacteriophage was tested as follows: Nutrient agar plates were streaked once with a large loopful of a suspension of the appropriate phage and allowed to dry. Broth cultures to be tested were then streaked across the phage inoculum. Plates were incubated for twenty-four hours and examined for lysis.

4. Preparation of Lambda Lysates: A twenty-four hour Pennassay broth culture of a lysogenic strain was centrifuged to sediment the cells. The supernatant fluid was retained and 0.1 ml of $CHCl_3$ was added to 5.0 ml of the fluid to kill any remaining cells. The mixture was allowed to stand at room temperature for twenty-four hours before use.

5. Tests for Lysogenicity: See section D, part 3 of Results.

E. Incubation of Cultures

All cultures were incubated at 37 C unless otherwise stated.

RESULTS

A. Serological Analysis of the Surface Antigens of CS11

Antiserum produced in response to formalin-killed whole-cell suspensions of the E. coli sub-strain, CS11, agglutinates cultures of the non-flagellated K12 x B hybrid, MSC56 (see Table II). Both MSC56 and CS11 are agglutinated by high (1:1280) dilutions of CS11 antiserum; but when the antiserum is absorbed with dense cell suspensions of MSC56 until this strain no longer is agglutinated, CS11 continues to react with 1:160 dilutions, suggesting that the antiserum contains two antibody components, one of which does not have an antigenic counterpart in MSC56. This, in turn, indicates that CS11 possesses at least two genetically separable antigenic components.

A motile K12 x B hybrid, MSC39, also is agglutinated by high dilutions of CS11 antiserum. Like MSC56, MSC39 also only partially absorbs CS11 agglutinins from antiserum prepared against CS11. Absorption by MSC39 is not nearly as effective as absorption by MSC56. Results given in Table II show that antiserum absorbed by MSC39 agglutinates CS11 at a 1:640 dilution. Nonetheless, the possibility arises that the two hybrids are antigenically alike. To test this possibility, antiserum was prepared with MSC39 and tested for its ability to agglutinate the three strains. Results given in Table II show that high dilutions of this antiserum agglutinated both CS11 and MSC39. MSC56 was not agglutinated even at a dilution of 1:20. These results demonstrated an antigenic relationship between MSC39 and CS11 but not between

Table II. Serological Relationships Among CS11, MSC39, and MSC56.

Test antigen	Reciprocal of agglutinating titer in CS11 antiserum: Unabsorbed maximum agglut.	Unabsorbed detectable agglut.	Absorbed with MSC39 maximum agglut.	Absorbed with MSC39 detectable agglut.	Absorbed with MSC56 maximum agglut.	Absorbed with MSC56 detectable agglut.
CS11	1280	2560	640	1280	80	160
MSC56	1280	2560	640	1280	0	0
MSC39	640	1280	0	0	160	320

	Reciprocal of agglutinating titer in MSC39 antiserum: maximum agglut.	detectable agglut.
CS11	640	1280
MSC56	0	0
MSC39	640	1280

"0" means negative in a serum dilution of 1:20. Control serum tubes were all 0.

MSC39 and MSC56. These relationships were clarified further when it was found that antiserum absorbed with MSC56 continued to agglutinate MSC39 and that antiserum absorbed with MSC39 continued to agglutinate MSC56. The ability of MSC56 to lower the agglutinating titer of CS11 antiserum for MSC39 is unexplained. When CS11 antiserum was absorbed sequentially with MSC56 and MSC39, it no longer agglutinated CS11. It was concluded that CS11 antiserum contains two antibody components, one of which corresponds to the flagellar antigen of MSC39, the other to an antigen carried by MSC56 and not clearly associated with flagella. Results of the following test show that the antigen of MSC56, when carried by CS11, is not intimately associated with the flagella. When motilized cultures are inoculated into the center of a plate of semi-solid culture medium, the cells will migrate through the medium producing a visible and increasingly larger circle of growth extending toward the periphery of the plate. If homologous flagellar antibody is incorporated into the medium, this migration of cells is impeded and growth is confined to the site of inoculation. To determine whether the antibody remaining in CS11 antiserum absorbed with MSC39 is associated with flagellar antigen, a soft agar plate containing a 1:50 dilution of this antiserum was inoculated with an actively motile culture of CS11. At the same time, a plate containing the same dilution of unabsorbed CS11 antiserum was similarly inoculated. A third inoculated plate containing normal serum served as a control. The culture swarmed through the medium containing the absorbed antiserum as well as through

that containing control serum. No swarming occurred in the medium containing unabsorbed serum. It is inferred from this data that the antibody remaining in the absorbed serum does not correspond to an antigen of structural CS11 flagella.

The flocculent nature of the MSC56 agglutination suggests that, as in "H" (flagellar) agglutination, the reacting antigen is situated at some distance from the main contour of the cell. In fact, the possibility arises that the antigen of MSC56 is a "secretion" not physically in contact with the cell. This situation seems unlikely in view of results obtained from the following experiment. A twenty-four hour Pennassay broth culture of a K12 sub-strain (MSC501) known to lack the antigen possessed by MSC56 (see later section) and a similar culture of MSC56 were centrifuged, decanted, washed in sterile broth, and resuspended in the reciprocal supernatant broth culture fluids. The cells of MSC56 resuspended in the supernatant fluid of the MSC501 culture were agglutinated by a 1:160 dilution of absorbed antiserum; no agglutination was seen in the suspension of MSC501 cells. Thus it appears that the MSC56 antigen is an adherent part of the cell surface.

B. Physical Properties of the Antigen of MSC56

The antigen-antibody aggregate formed by MSC56 and by CS11 in response to antiserum is very similar to, but not identical with, that produced by MSC39. In both cases, the response is rapid; agglutination usually is visible in the first tube within ten minutes and is complete within an hour. Both give the flocculent appearance characteristic of

flagellar agglutination but the aggregate formed by MSC39 is very fragile and easily dispersed by shaking. The aggregates formed by MSC56 and by CS11 are not completely dispersed by shaking but remain suspended as discrete particles which are readily visible when viewed under the dissecting microscope.

Weibull (1949) has done an extensive chemical analysis of the flagella of some of the Enterobacteriaceae and found them to be mainly, if not completely, protein. The antigenic stability of the flagella of this group is described (Cruickshank, 1960) as being destroyed at temperatures of from 80 C to 100 C for about twenty minutes. This heat lability, along with the flaky nature of the agglutination, is characteristic of the flagellar antigen-antibody reaction and serves to distinguish it from other serological reactions commonly observed for the surface antigens in this group of bacteria. If the antigen of MSC56 is, in fact, a flagellar protein, it should be heat labile like the antigen of structural flagella. This point is of more than incidental interest for the following reason. Motile strains of E. coli, unlike the closely-related Salmonella, are monophasic. No strains have been described which have more than one flagellar serotype. It may be, however, that E. coli is normally diphasic, one phase represented by a unique state in which flagellar protein is present but not organized as structural flagella. This would be consistent with the failure of standard methods to detect a typical diphasic condition.

Ørskov and Ørskov (1960) reject the K12 and B strains of E. coli

as suitable subjects for serological study of surface antigen on the basis that they are auto-agglutinable. Maccacaro and Colombo (1956) report the detection of K and O antigens in K12 strains using saline suspensions of cells in agglutination tests designed to compare antigenic properties of F- and F+ cultures. Our experience has been that cultures suspended in saline are readily auto-agglutinable especially upon heating. Similarly, slide agglutination tests using growth from solid media suspended in saline have been found to be unreliable. However, by means of tube agglutinations carried out with Pennassay broth cultures, it has been possible to obtain reliable serological data. The heat stabilities of CS11, MSC39, and MSC56 were examined by this method. Results appear in Table III.

All three strains were heated to 71 C for fifteen minutes and subsequently tested for reaction with antiserum. CS11 and MSC56 continued to be agglutinated by 1:640 dilutions of CS11 antiserum. MSC39 showed no agglutination with CS11 antiserum. Neither MSC39 nor CS11 were agglutinated by MSC39 antiserum even at a 1:20 dilution. As the cultures of CS11 and MSC56 were exposed to increases in temperature, their ability to be agglutinated declined. Both, however, gave a strong reaction in a 1:20 dilution of antiserum even after having been heated to 110 C for an hour. No appreciable difference in agglutinability was noted for any given temperature if the time of heating was increased from fifteen minutes to an hour. For example, CS11 reacted strongly with antiserum at a dilution of 1:80 when heated to

Table III. Heat Stability of Antigens.

Test antigen	Reciprocal of agglutinating titer of: CS11 antiserum maximum agglut.	CS11 antiserum detectable agglut.	MSC39 antiserum maximum agglut.	MSC39 antiserum detectable agglut.
Unheated				
CS11	1280	2560	640	1280
MSC39	640	1280	640	1280
MSC56	1280	2560	0	0
71 C, 15 min				
CS11	640	1280	0	0
MSC39	0	0	0	0
MSC56	640	1280	0	0
82 C, 1 hr				
CS11	320	640		
MSC56	320	640		
87 C, 15 min				
CS11	160	640		
MSC56	320	640		
100 C, 15 min				
CS11	80	640		
MSC56	80	640		
100 C, 1 hr				
CS11	80	160		
MSC56	80	160		
110 C, 1 hr				
CS11	20	40		
MSC56	20	40		

"0" means negative in a serum dilution of 1:20. Control serum tubes were all 0.

100 C for fifteen minutes and reacted to approximately the same dilution after heating for a full hour.

These experiments indicate clearly that the antigens of MSC39 and MSC56 have distinct and different heat stabilities. The behavior of the MSC39 antigen is completely compatible with the accepted criterion for "H" antigenicity. That of MSC56 is not. It is necessary, therefore, to exclude the hypothesis that the MSC56 heat stable antigen is a non-functioning flagellar antigen.

In subsequent sections, this antigen will be referred to as the Heat Stable Antigen (HSA) and CS11 antiserum absorbed with MSC39 as HSA antiserum.

C. Occurrence of HSA Among K12 Strains

Since no antigen like HSA has been described for E. coli K12, it was appropriate to determine whether CS11 was an unusual antigenic mutant of the strain. A survey was made from among the stock cultures in our collection. Nine sub-strains were examined for presence of the antigen as well as for all other known markers. Results are shown in Table IV. The following seven cultures reacted with HSA antiserum and failed to agglutinate when tested quantitatively with control serum: MSC503, CS11, MSC69, MSC70, W6, CS19, and MSC18. The Hfr strain, CS101, is agglutinated but gives a less striking reaction with antiserum than do the cultures cited above. Another Hfr culture, W1895, agglutinates spontaneously both in test serum and in control serum. MSC501 alone is stable in control serum and fails to react with antiserum, and is

Table IV. HSA Status of Selected K12 Strains.

Strain	Auxotrophic markers	Other markers							HSA agglutination*	
		V_1	lac	mal	xyl	mtl	S	sex	maximum agglut.	detectable agglut.
CS11	A^-, T^-, L^-, B_1^-	R	–	–	–	–	R	F+	640	1280
MSC503	T^-, L^-, B_1^-	R	–	+	–	–	S	F–	640	1280
MSC501	T^-, L^-	R	–	+	–	+	R	F–	0	0
W6	M^-	S	+	+	+	+	S	F+	1280	2560
CS19	pa^-	S	+	+	+	+	S	F–	640	1280
MSC18	M^-	S	+	+	+	+	S	F+	640	1280
MSC70	M^-	S	+	+	+	+	S	F+	640	1280
MSC69	M^-	S	+	+	+	+	S	F–	320	640
CS101	M^-	S	+	+	+	+	S	Hfr	320	640
W1895	M^-	S	+	+	+	+	S	Hfr	A	A

*Numbers given in this column represent the reciprocal of the highest dilution of antiserum which gives agglutination. "A" in this column means auto-agglutination. All other serum controls were negative. "0" in this column means negative in a serum dilution of 1:20.

R or S indicate resistance or sensitivity to streptomycin (column S) or to T5 bacteriophage (column V_1). + or – indicate ability or lack of ability to ferment the following carbohydrates: lactose, maltose, xylose, mannitol. Notation for auxotrophic markers is as follows: A, arginine; L, leucine; T, threonine; B_1, thiamine; M, methionine; pa, phenylalanine.

hence HSA$^-$.

As can be seen from Table IV, no obvious correlation exists between the HSA property and possession of any other known marker. No correlation between colonial morphology and HSA has been observed. Some of the HSA$^+$ strains consistently produce rough colonies while others produce smooth colonies. Possession of HSA can not be correlated with the mucoid appearance which K12 cultures often present. CS11, for example, produces large amounts of mucus and consistently reacts with high dilutions of HSA antiserum. MSC501 also tends to produce large amounts of mucus but does not react with HSA antiserum even when test antigens are prepared from particularly mucoid growth. Pennassay broth cultures of HSA$^+$ strains typically produce an adherent residue around the inside of the test tube which can be demonstrated by tilting the culture tube to displace the broth from the side of the tube. The presence of this residue is reliable enough to serve as a convenient screening device in searching for HSA$^+$ cultures. It is not infallible however. Broth cultures of some strains which produce rough colonies on solid medium are often clear with most of the cells sedimented at the bottom of the tube. These broth cultures do not produce the adherent residue characteristic of HSA$^+$ strains but may give typical agglutination when tested with antiserum.

The results of this limited survey of strains clearly shows that possession of HSA is not a unique property of CS11 but is characteristic of many K12 sub-strains and, further, that this antigenic

property is not a pleiotropic effect of any of the known genetic markers. The consistent and uniform reaction of most cultures when tested with antiserum shows that HSA is a stable characteristic not readily influenced by variation in genetic or environmental background. That the HSA$^-$ property of MSC501 is likewise stable is inferred from two years observation of this strain. Even more dramatic evidence of the stability of HSA$^-$ was provided by examination of a new culture of W1177 provided by Dr. J. Lederberg. MSC501 is a culture of W1177 which, in the course of storage in Dr. Skaar's laboratory, has become mal$^+$, mtl$^+$, and $B_1{}^+$. The Lederberg culture of W1177 has acquired a new requirement for phenylalanine. In spite of ten years isolation, these two W1177 derivatives are both HSA$^-$. Infrequent reversions of HSA$^-$ to HSA$^+$ and the reverse among variants of W1177 and MSC501 will be the subject of the next section.

D. Mutation of the HSA Property in Derivatives of W1177

The HSA$^-$ nature of MSC501 has been known for about two years. During this period the culture has been propagated on nutrient agar slants and has been repeatedly subcultured. Broth cultures inoculated from the agar slants have consistently failed to react with HSA antiserum; however, on one occasion, when broth cultures were diluted and plated on solid medium to give isolated colonies which, in turn, were transferred to broth, a few HSA$^+$ revertants were detected. All of these revertants arose on plates of EMB lactose which had been spread with high dilutions of one Pennassay broth culture of MSC501. Five of

forty colonies picked were found to be HSA$^+$. All of these cultures have been retained on nutrient agar slants and continue to be stable for the HSA$^+$ property and give reactions in antiserum which are comparable with that of CS11. One of these cultures is MSC505.

It seems unlikely that growth on EMB lactose had induced the change in antigenicity; but, to test this possibility, another inoculum from the same MSC501 slant was grown up in broth, diluted, and plated on EMB lactose. A second plating was made from an HSA$^+$ revertant. Forty colonies from each plating were examined for mutation of HSA. No mutation in either direction was observed. Obviously, this small sampling is of little value in determining the rate of mutation of the HSA character, but the results strengthen our impression that the change is rare and occurs with a frequency that is more compatible with a true mutation than with a more narrowly oscillating change such as phase variation.

Historically, W1177 was obtained by Dr. Lederberg as a streptomycin resistant mutant of W677. Our culture of W677 (which has been renumbered MSC503 because of a mutation from mal$^-$ to mal$^+$) is stably HSA$^+$. Since the culture of W1177 recently obtained from Dr. Lederberg gives an HSA$^-$ reaction, it is believed that the HSA$^+$ character was lost simultaneously with the loss of streptomycin sensitivity. CS11 is a derivative of W1177 which was made F+ by Dr. P. D. Skaar by treatment with strain 58-161. CS11 consistently gives an HSA$^+$ reaction. Thus it appears that the HSA$^+$ to HSA$^-$ change may have accompanied the mutation

to streptomycin resistance and that the HSA$^-$ to HSA$^+$ reversion may have accompanied the acquisition of the sex factor.

In addition to the W1177 culture, two derivatives of W1177 which differ in their properties relating to the temperate bacteriophage, lambda, were obtained from Dr. Lederberg and tested for their reaction with HSA antiserum. The lambda-related properties of K12 strains are controlled by genes at two loci. The Lp1 locus controls possession of the prophage, the Lp$_2$ locus controls possession of the receptors for adsorption of phage (Lederberg and Lederberg, 1953). Lp$_2^r$ cultures cannot adsorb lambda phage and are therefore immune to infection even though they may be non-lysogenic. Lp$_2^s$ cultures are able to adsorb phage and, if non-lysogenic, are sensitive to infection by extrinsic phage. The genotype of the HSA$^-$ strain, W1177, is Lp$_1^+$, Lp$_2^r$. W1603, which is an Lp$_1^-$, Lp$_2^r$ derivative of W1177, is HSA$^+$. W2736, which is an Lp$_1^-$, Lp$_2^s$ derivative of W1603 is HSA$^-$. The appearance, in rapid succession, of two reversions in the HSA property occurring simultaneously with mutational changes in the lambda properties raises the possibility that changes of the HSA state may be invariably accompanied by changes of other, not obviously related, properties.

All of the five HSA$^-$ to HSA$^+$ revertants of MSC501 were different from the parent strain when grown on EMB mannitol. When MSC503 is grown on EMB mannitol, the colonies which arise have a typical mtl$^-$ appearance. The appearance of MSC501 colonies on EMB mannitol is intermediate between mtl$^-$ and mtl$^+$. Each of the five revertants gave a

typical mtl$^-$ reaction. The fact that all nine of the observed mutations of the HSA character have occurred simultaneously with other genetic changes affecting properties which have no obvious physiological relationship to HSA or to each other made it seem appropriate to determine whether the concurrent mutations were simply fortuitous events. Because of the lack of suitable methods for selecting HSA mutants, the problem was approached by studying the associated mutations. The changes in colonial appearance on EMB mannitol was not investigated because this mutation also does not lend itself to selective methods. The changes in streptomycin resistance, lambda characteristics, and mating properties (gain or loss of sex factor) can be readily studied.

1. Mutation to Streptomycin Resistance: Chronologically, the first observed mutation to HSA$^-$ appeared at the time of origin of W1177. The first possibility to be considered is that HSA$^-$ and S^r are pleiotropic effects of a single gene. This is ruled out by the occurrence of HSA$^+$ derivatives of MSC501 which are still S^r. A remaining explanation is that, in the genetic background of MSC503 (HSA$^+$, S^s), mutation to HSA$^-$ always accompanies the S^s to S^r mutation. To test this second possibility, new streptomycin resistant mutants of MSC503 were sought by the following procedure. One-tenth ml quantities of an overnight Pennassay broth culture of MSC503 were transferred to forty 5.0 ml quantities of Pennassay broth and incubated twenty-four hours at 37 C. These cultures were centrifuged and the pellet resuspended in broth containing streptomycin and reincubated. Four of the

forty tubes showed turbid growth within forty-eight hours. These cultures were streaked on nutrient agar containing streptomycin and incubated until well-developed isolated colonies appeared which were then picked and transferred to tubes of Pennassay broth. After twenty-four hours incubation, the cultures were tested for HSA and for streptomycin resistance. All were found to be $\underline{HSA}^+$ like MSC503 and were streptomycin resistant. Thus it is evident that the mutation to streptomycin resistance and the mutation to $\underline{HSA}^-$ are not invariably associated in MSC503. Obviously, the small number of mutants examined in this experiment offers little indication of the frequency of similar simultaneous mutations among K12 strains in general. To obtain a larger sampling of mutants, the same selection was made using strain CS19. CS19 is a sub-culture of the mutable strain, 58-278. This culture mutates from streptomycin sensitivity to streptomycin resistance at a frequency which is about a hundred times greater than that of other K12 strains (Treffers, et al, 1954; Skaar, 1956). Among 100 such streptomycin resistant mutants obtained, all were found to be $\underline{HSA}^+$. Thus it appears that the HSA mutation occurs at a frequency which is less than one per one hundred mutations to streptomycin resistance.

If the genetic basis for the HSA property is cytoplasmic, the simultaneous occurrence of $\underline{S}^r$ and $\underline{HSA}^-$ mutations might be attributed to the streptomycin treatment employed in selecting the $\underline{S}^r$ mutant, W1177. The ability of streptomycin to disinfect certain cells of cytoplasmic genes has been demonstrated in algal flagellates (Provasoli, et al,

1951), in seed plants (Bogorad, 1950), and in Euglena (Provasoli, et al, 1948). In order to test this possibility, cultures of MSC500, which is a streptomycin resistant variant of MSC56, and of CS11 were subcultured daily for fourteen days in Pennassay broth containing streptomycin. The cultures were tested daily for reaction with HSA antiserum. Both cultures continued to react with high dilutions of antiserum which meant that they were not massively converted to an HSA$^-$ state.

2. Conversion of F- Strains to F+: Genetic recombination by conjugation in E. coli depends upon a sexual differentiation of the strains involved. Genes are transferred uni-directionally from an F+ donor to an F- recipient. This difference is based on the presence of a sex factor which is possessed by F+ strains but not by F- strains. The sex factor is independent of the bacterial chromosome and can be transferred at high frequency from an F+ to F- culture, thereby conferring upon the latter the ability to act as a gene donor (Cavalli, et al, 1953).

The sex factor is assumed to have two effects upon the cell it inhabits. The first effect is to bring about a surface change which facilitates pairing with compatible F- cells; the second is to effect the migration of chromosomal genes (Mäkelä, et al, 1962). It has been proposed that, since unlike cell metabolites, enzymes, or vegetative phage, the sex factor is transferred at high frequency in F+ x F- crosses, the sex factor structure is not randomly distributed in the cytoplasm but occupies a specific site close to the bacterial cell wall

(Jacob, et al, 1960).

Certain differences between the physical properties of F+ cell surfaces and those of F- cells have been reported by Maccacaro (1955). Sneath and Lederberg (1961) have found that treatment of F+ cells with metaperiodate makes them phenotypically unable to act as gene donors, presumably by the oxidation of surface carbohydrates which function in the pairing process. Differences between F- and F+ cultures of K12 strains with respect to their agglutinability with homologous antiserum have been reported by Maccacaro and Colombo (1956).

Ørskov and Ørskov (1960) report an "f^+" antigen which occurs in strains of E. coli which have been converted to the donor state by treatment with F+ K12 strains. All converted strains tested were shown to possess the f^+ antigen but not all $(f^+)^+$ strains were found to be F+. Some strains of E. coli have, in fact, been found to have the capacity for converting other strains to the F+ state even though they themselves could not act as donors for chromosomal genes (Furness and Rowley, 1957). The Ørskovs hypothesize a "defective" sex factor which fulfills the function of pairing and of antigenicity but not of promoting gene transfer.

It is clear that not all F- strains are $\underline{HSA}^-$. MSC503 which is F- is $\underline{HSA}^+$ and is able to absorb all detectable antibody from the homologous antiserum of the F+ strain, CS11 (see Table V). However, the fact that MSC501 (F-, $\underline{HSA}^-$) when made F+ (CS11) also became $\underline{HSA}^+$ raises the possibility that the HSA antigen is indispensable (but not

Table V. Serological Relationships Among F-, F+, and Hfr Strains.

Test antigen	Reciprocal of agglutinating titer in CS11 antiserum: absorbed with MSC39	absorbed with MSC503	absorbed with CS101
CS11 F+	640	0	0
MSC503 F-	640	0	0
CS101 Hfr	640	0	0

"0" means negative in a serum dilution of 1:20. Control serum tubes were all 0. Figures are for maximum agglutination.

sufficient) for the mating process. On this basis, all F+ strains should be HSA$^+$. To test this, F+ derivatives of MSC501 were obtained by mixed growth with two F+ donors, MSC70 and W6. Overnight cultures of the three strains were grown up separately in Pennassay broth to a concentration of about 1 x 10^9 cells per ml. One hundred fold dilutions were made of all cultures and they were incubated again until they reached concentrations of about 1 x 10^8 cells per ml. Then, 2.5 ml quantities of MSC501 were added to 5.0 ml quantities of each of the F+ cultures, and the mixtures were incubated in the 37 C water bath for one hour. Dilutions were made of the cultures and 0.1 ml samples were spread on plates of EMB lactose to give approximately 100 colonies per plate. The plates were incubated at 37 C for 30 hours. Well-isolated lac$^-$ colonies were transferred to Pennassay broth, incubated 16 hours at 37 C and then crosses were made with IMN64 on DMA plates with selection for transfer of the shikimic acid locus. The DMA plates were incubated for 48 hours at 37 C and scored for the number of prototrophic recombinants. Seven cultures of MSC501 from the mixing with W6 gave large numbers of recombinants with IMN64. Four cultures of MSC501 from the mixing with MSC70 also gave large numbers of recombinants. Control platings of the cross components alone were barren. None of the broth cultures from the newly-transformed MSC501 F+ strains reacted with HSA antiserum, indicating that the HSA antigen is not an essential attribute of F+ K12 strains.

To test the possibility that HSA$^+$ cells might differ from HSA$^-$

cells in the readiness with which they are converted to F+, the following experiment was performed. Cultures of three F- strains (MSC501, MSC503, and MSC505) were exposed to W6 as in the preceding experiment, and the purified progeny of the exposed cells were tested for F status. MSC503 (the mal$^+$ derivative of W677) is HSA$^+$; MSC501 (the mal$^+$ derivative of W1177) is HSA$^-$; MSC505 is the HSA$^+$ derivative of MSC501. As seen in Table VI (First Cross), all three strains were converted to F+ with equal facility.

To determine whether the F+ state was stable in the newly converted strains, each was streaked to a second set of EMB lactose plates. These plates were incubated 30 hours as before and broth cultures derived from isolated colonies were tested for their ability to act as genetic donors for IMN64 (Second Cross, Table VI). In all cases, the F+ property of the newly converted strains was retained in the subculture. No explanation can be offered at the present time for the inferior fertility of MSC501 and MSC505 derivatives, as contrasted with those of MSC503.

Again, all newly converted F+ derivatives of MSC501 were still HSA$^-$ at the time of both the first and second crosses.

The preceding results clearly show that K12 strains may be F+ without HSA. In addition, they show that, if HSA is episomally determined, the infectivity of that episome is lower than is that of the sex factor.

3. HSA Mutations Associated with Changes in Lambda Related

Table VI. Relative Efficiency of F+ Conversion of MSC501, MSC503, and MSC505 by Mixed Growth with W6.

	Prototrophic recombinants per plate in crosses with IMN64:					
	First cross			Second cross		
Treated strains	MSC501	MSC505	MSC503	MSC501	MSC505	MSC503
Extracted clones						
1	100	0	0	100	0	0
2	100	100	>1000	200	>1000	>1000
3	40	160	>1000	100	200	>1000
4	100	0	0	60	0	0
5	0	100	0	0	400	0
6	0	0	0	0	0	0
7	0	300	0	0	400	0
8	70	100	>1000	100	120	>1000
9	0	0	>1000	0	0	>1000
10	500	140	>1000	200	200	>1000

Cultures of MSC501, MSC505, and MSC503 (all *lac*$^-$) were grown with W6 (*lac*$^+$). Ten *lac*$^-$ clones were then extracted from each mixture. These were immediately tested for F status by crossing to IMN64 (first cross). Each clone was streaked and a new culture initiated from an isolated colony. This new culture was then tested for F status (second cross). A control cross between W6 and IMN64 gave >1000 prototrophic recombinants. Control platings of all components were negative.

Properties: W1177 is HSA$^-$, Lp$_1^+$, Lp$_2^r$, and mal$_1^-$. The corresponding mal$^+$ strain, MSC501, is HSA$^-$, Lp$_1^+$, and presumably Lp$_2^s$ according to Lederberg's conclusions that Lp$_2^s$ and mal$_1^-$ are under the pleiotropic control of a single gene (Lederberg, 1955). W1603 is HSA$^+$, Lp$_1^-$, Lp$_2^r$, mal$_1^-$. Its derivative, W2736, is HSA$^+$, Lp$_1^-$, Lp$_2^s$, mal$_1^+$.

The HSA character and the properties related to lambda are not under pleiotropic control since W1177 and W2736 are alike for HSA but different for Lp$_2$ while W1177 and W1603 are alike for Lp$_1$ but different for HSA.

To determine whether a mutation at either the Lp$_1$ or the Lp$_2$ locus can be correlated with changes in the HSA property, mutants of W2637 were sought for study by the following method: A nutrient agar plate was spread with a 0.1 ml overnight Pennassay broth culture of W2637 and allowed to dry. A lambda suspension was prepared from MSC503 (see Materials and Methods) and a loopful of this suspension was spotted on the W2637 plate.

The plate was incubated for 24 hours and examined for lysis. A lawn of growth appeared over the entire plate except for an area of nearly confluent lysis in the region where the phage had been applied. Streakings were made from this lysed area to several plates of EMB maltose which had been spread with streptomycin to kill any contaminating MSC503 cells. The plates were incubated for 24 hours. Fifty-four colonies were picked and transferred to tubes of Pennassay broth, incubated for 24 hours and tested with a 1:40 dilution of HSA antiserum.

None were agglutinated by the antiserum.

Among twenty-eight of the colonies picked from the maltose streptomycin plate, twenty-one were *mal*$^+$ and immune to *lambda*. Fifteen of these were demonstrated to be lysogenic. The remaining colonies were *mal*$^-$ and (surprisingly) sensitive to *lambda*. The appearance of *mal*$^-$ *lambda* sensitive mutants is interesting in itself and deserves further study. The important point here, however, is that mutation of the HSA property is not invariably correlated with mutation from *lambda* sensitivity to *lambda* lysogenicity nor with the mutation from *mal*$^+$ to *mal*$^-$.

E. Mutation of HSA Associated with Enhanced Motility

The loss of fertility associated with prolonged passage on motility medium has been interpreted as due to selection for alleles with pleiotropic effects on fertility and rapidity of swarming (Skaar, *et al*, 1957). These authors suggest that cells not encumbered by a surface component necessary for fertility may be thus fortuitously selected as rapid swarmers. For the same reason, it might be predicted that selection for high motility might result in correlated selection for *HSA*$^-$. This possibility was tested as follows: A culture of W6 was motilized by ten successive transfers on motility medium until the entire population was highly motile and gave a delicate swarm. An inoculum from the edge of the swarm on the tenth plate was transferred to Pennassay broth and incubated. At the same time, an inoculum from the stock culture agar slant was similarly transferred to broth and

incubated. At the end of forty-eight hours incubation the broth cultures were tested quantitatively for agglutination by HSA antiserum. The culture derived from the agar slant was agglutinated strongly by a 1:640 dilution of antiserum; the highly motile culture showed no detectable agglutination even with a 1:20 dilution of antiserum. The broth cultures were then subcultured into fresh broth and a third broth culture was initiated from an inoculum obtained directly from a swarm on motility medium. All three cultures were tested for their ability to produce prototrophic recombinants with W1177. The method used in this cross was identical with that described in section F of Results except that the DMA plates were spread with phenylalanine (see Materials and Methods) to relax selection for transfer of the $\underline{pa}^+$ gene.

After two days incubation, the DMA plates were scored for prototrophic recombinants. The non-motilized culture gave more than 1000 recombinants, the cross with the first motilized culture showed an approximately ten-fold decrease in number of recombinants, and the culture which was inoculated directly from the swarm showed an approximately twenty-fold decrease in number of recombinants. These results suggest that loss of $\underline{HSA}^+$ and loss of F+ may accompany the selection of the highly motile genotype. The results also show, however, that $\underline{HSA}^+$ and F+ do not have a common genetic basis, supporting conclusions reached in a previous section.

F. Localization of the HSA Gene on the Bacterial Chromosome

Strain W1177 was isolated as a streptomycin resistant mutant of

W677. MSC501 is a mal$^+$ mutant of W1177 and MSC503 is a mal$^+$ mutant of W677. MSC503 is agglutinated by a 1:640 dilution of HSA antiserum. Agglutination at this dilution is strong; that is, a large flaky antigen-antibody aggregate is formed and the supernatant liquid is clear. MSC501 gives no visible reaction with HSA antiserum and absorbs only flagellar antibody from CS11 antiserum. The agglutination of HSA$^+$ revertants of MSC501 is indistinguishable from that of MSC503. These facts suggest that the genetic basis of the HSA differences among the strains resides at a single locus. If this is so, and if the HSA locus is chromosomal, it should be possible to establish the position of the locus by standard crosses. With this end in view, a cross was made between W6 (F+ HSA$^+$) and W1177 (F-, HSA$^-$).

The cross was made on minimal medium with selection for prototrophy. W6 is deficient for methionine, W1177 is deficient for threonine, leucine, and thiamine. A large number of prototrophic colonies appearing on the cross plates were streaked on DMA; then single colonies from these plates were streaked on complete medium. Isolated colonies from the complete medium were transferred to 5.0 ml quantities of Pennassay broth and incubated for twenty-four hours. These broth cultures were tested for agglutination by 1:40 dilutions of HSA antiserum, and for auto-agglutination in identical dilutions of control serum. At the same time the broth cultures were verified as prototrophs by spotting on plates of DMA and were also tested for six unselected markers involved in the cross. Results are given in Table VII. The data of Lederberg,

Table VII. Segregation of HSA and Other Unselected Markers Among Prototrophic Recombinants Resulting from Crosses Between W6 (F^+, V_1^s, lac^+, xyl^+, mal^+, mtl^+, S^s, HSA^+) and W1177 (F^-, V_1^r, lac^-, xyl^-, mal^-, mtl^-, HSA^-).

A. Frequency of Transfer of F+ Alleles

	Total number examined	% transfer of F+ alleles of:						HSA status of* recombinants	
		V_1	lac	xyl	mal	mtl	S	HSA+	HSA±
Markusen (1963)	106	73	42	10	9	8	15	16	12
Lederberg et al (1951)	100	67	28	---	21	17	15	---	---

B. Relationship Between the Transfer of HSA^+ and the Transfer of Other Unselected F+ Alleles

HSA status*	Total number examined	% transfer of F+ alleles of:					
		V_1	lac	xyl	mal	mtl	S
+	17	76	53	12	12	6	12
±	13	77	62	23	15	23	31
-	76	71	36	8	8	7	13

*In these columns, + means strong agglutination in a 1:40 dilution of HSA antiserum, ± means partial agglutination at the same dilution of antiserum.

et al (1951) are included in part A to show that the proportion of unselected markers observed in the present cross is distorted somewhat from that usually obtained in a cross between an F+, M^- strain and an F-, T^-, L^-, B_1^- strain. Specifically, the frequency of lac transfer is higher than expected, while the frequency of transfer of mal and mtl are lower than expected. The distortion may fall within the range of variation among different crosses. On the other hand, it may be due to the complication that W1177 has acquired an additional nutritional requirement for phenylalanine. The phenylalanine locus (or loci) have not been well studied. One locus is known not to be closely linked to TLB_1 (Jacob and Wollman, 1961). The high incidence of lac^+ appearing among the recombinants suggests that the new pa mutation is near lac. Since the frequency of V_1^s (between TLB_1 and lac) is not notably high, it is unlikely that pa is similarly situated.

On this basis, the frequency of HSA^+ prototrophs indicates that the responsible locus for HSA is either in the vicinity of TLB_1 or of the pa locus. However, before discussing this point further, another unexpected feature of this cross needs to be considered. This was the appearance of four recombinant populations which were heterogeneous for the lac marker. Recombinants obtained from K12 x K12 crosses usually are stable. The merozygote quickly segregates and gives rise to stable haploid clones. Exceptions have been observed; occasional crosses produce persistent heterozygotes (Lederberg, 1949). The detection of segregating lac^+ clones in the present cross, in spite of

standard precautions of recombinant purification, reveals another important complication also attributable, perhaps, to the new pa marker.

Twenty-eight per cent of the prototrophic recombinants were agglutinated by a 1:40 dilution of HSA antiserum; however, they did not react uniformly as was expected. Seventeen cultures gave a strong reaction like that of MSC503. The remaining thirteen were definitely agglutinated, but in these the supernatant liquid was not clear. No cultures agglutinated in control serum. The partial agglutination could be explained if some recombinants had received only a portion of an HSA complex, but this explanation is incompatible with our observations on the HSA⇌HSA$^+$ mutations and with the uniformity of the HSA$^+$ character of strains having very diverse genetic backgrounds (see Table IV). Another explanation is that the clones with which some of the broth cultures were inoculated were heterogeneous for HSA. This possibility is strengthened by the presence of clones described above which were mixed for lac.

Existence of a new selected marker plus the occurrence of segregating diploids confuses attempts to interpret the data with regard to location of the HSA gene. One important statement can be made with confidence: If the HSA difference between W6 and W1177 is due to a single locus, that locus does not lie in the segment of the Jacob-Wollman circular linkage map extending from B_1 to lac (see Figure 1). Transfer of the linked block of genes, B_1^+, T^+, L^+ is obligatory in this cross. Any selected markers lying between B_1 and L, or closely to

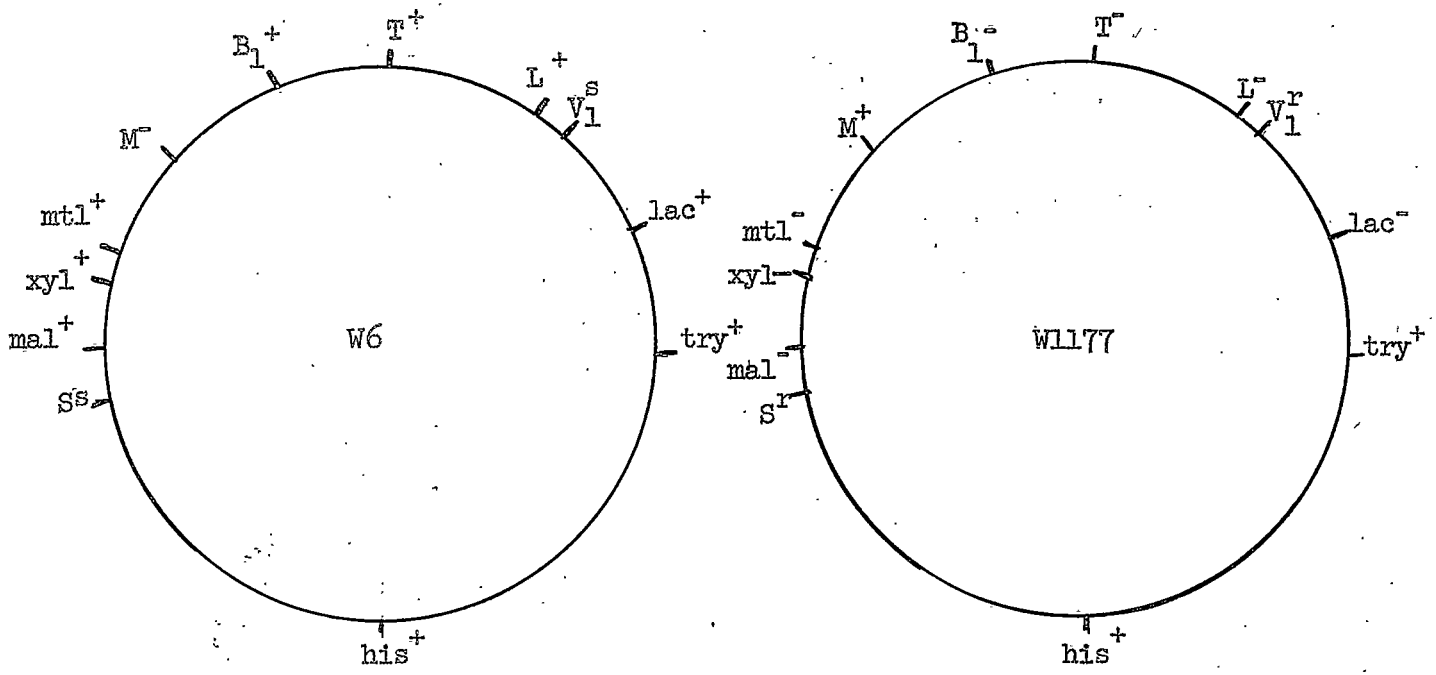

Figure 1. Genomes of W6 and W1177 Based on the Linkage Map of Jacob and Wollman (1961). Symbols used correspond to synthesis of threonine (T), thiamine (B_1), leucine (L), tryptophan (try), histidine (his), methionine (M); sensitivity or resistance to streptomycin (S), sensitivity or resistance to T5 (V_1); fermentation of maltose (mal), xylose (xyl), mannitol (mtl), lactose (lac).

the right, will appear in the prototrophic recombinants in high frequency. The genes, $\underline{V_1}^s$ and $\underline{lac}^+$, known to lie closely to the right of L and in that order, illustrate this. Even if both those recombinants giving a strong reaction with HSA antiserum and those giving only a partial reaction are scored as instances of passages of the F+ HSA allele, the percentage transfer of that allele (28) does not equal the percentage transfer of $\underline{lac}^+$ (42). Furthermore, the only possible complication of the new F- selective marker, $\underline{pa}^-$, (regardless of its location) would be to have increased the frequency of transfer of $\underline{HSA}^+$. This negative statement concerning the location of HSA is important, since the locus determining the somewhat similar fimbrial antigen has been located in the T L B_1 region (Maccacaro, et al, 1959).

Beyond this, only tentative statements can be made. Single colony isolates of W1177 alone have all proved to be $\underline{HSA}^-$, but such tests have been limited. Furthermore, no such examination was made of the same culture used in the cross. It is conceivable, therefore, that the crossed culture was a "jackpot" population containing a large number of $\underline{HSA}^+$ mutants. If so, the cross provides no evidence that the gene is even transferred by conjugation.

However, on the preceding hypothesis, the distribution of $\underline{HSA}^+$ cells should occur among the recombinants randomly with respect to other unselected markers. As shown in Table VII, there is a suggestion that this is not so, that, in fact, an interaction with lac exists. The data on $\underline{HSA}^+$ recombinants alone is insufficient to warrant confi-

dence in this interaction. If the frequency of *lac*$^+$ among both strongly agglutinating and partially agglutinating recombinants is compared to the frequency of *lac*$^+$ among the *HSA*$^-$ recombinants, however, the interaction is significant at the 5 per cent level ($X^2 \cong 5$).

If this interaction is real, it shows not only that *HSA*$^+$ is transferred, but also that, if the *HSA* locus is chromosomal, it lies somewhere to the right of *lac*. If the new *pa*$^-$ gene is responsible for the excess of *lac*$^+$ among all recombinants, it also would appear to lie to the right of *lac*, apparently beyond *HSA*. But *S* is not closely linked to *pa*, *pa* is not closely linked to *HSA*, nor is *HSA* closely linked to *lac*. Tentative positioning, therefore, would place *pa* near *his*[1], *HSA* near *try*[2] (see Figure 1).

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[1]Refers to the marker for histidine.

[2]Refers to the marker for tryptophan.

DISCUSSION

The role of HSA in the cell is difficult to ascertain. Its physical properties do not permit assigning it to any of the categories of the classical and well-studied O, K, and H antigens of E. coli. The somatic or O antigen is heat stable. Its antigenicity is not destroyed by heating to 100 C for two hours. The agglutinating reaction is slow and the aggregate produced is granular and firm. The H or flagellar antigen forms a loose, floccular aggregate but the antigen is heat labile (Kauffman, 1951). Its antigenicity, according to Mackie and McCartney (Cruickshank, 1960) is destroyed by heating to 60 C for about twenty minutes.

The K antigens are a group of envelope or capsular antigens which are responsible for the O-inagglutinability of living bacteria. The L antigen of this group is destroyed by heating to 100 C for an hour. The capsular A antigen is thermostable but is agglutinated only by low (1:10 or less) dilutions of antiserum. The B antigen occupies an intermediate position in this group. Its agglutinating antigen is heat labile but its antibody-binding capacity resists heating to 100 C for two and one-half hours. The M (mucus) antigen also is agglutinated only by low dilutions of antiserum. Its agglutinability is destroyed by heating to 100 C for two and one-half hours (Kauffman, 1951).

Ørskov and Ørskov (1960) describe the f^+ antigen as being destroyed by heating to 100 C for an hour. The agglutinating reaction is slow and both the appearance of the aggregate formed and the strength of the

agglutination are variable depending upon environmental factors. No attempt was made, during the course of the present investigation, to demonstrate a K12 antigen comparable to f^+. Falkow and Baron (1962) report agglutination of K12 F+ and Hfr strains by f^+ antiserum but no data are given concerning the source of the antiserum.

Many of the Enterobacteriaceae possess fimbriae (pili). Agglutination of fimbriated cultures by antiserum is similar to flagellar agglutination and is a common source of confusion (Cruickshank, 1960). The fimbrial antigens are more heat stable than are flagella but are destroyed by heating to 100 C for an hour (Gillies and Duguid, 1958).

Investigations into the genetic basis of surface antigens of those bacterial strains which lend themselves to standard recombination studies have been very limited. Linked transfer of genes for flagellar structure, function, and antigenic specificity in crosses between E. coli K12 mot^+ and E. coli B mot^- has been reported by Furness and Rowley (1955). Results from such crosses revealed a locus for the motility gene near a tyrosine marker (Furness, 1958). Phage-mediated transfer of motility between K12 and B strains (Markusen, 1960) also indicates that in these strains, all attributes of motility may be controlled by a single gene or by closely linked genes. In a cross employing another non-flagellated strain and using a K12 strain as the motile donor, Ørskov and Ørskov (1962) report the transfer of a fla gene to be independent of genes for antigenic specificity. They place the site of this gene in the his region. Transfer of a gene for O antigen was

demonstrated as well as those for two of the K antigens. Their data indicate that the gene for the O antigen is situated close to the his locus as is the H gene described above. According to the Ørskovs, markers for both the A and B types of K antigen are closely linked to the O locus but are separable from it. The L antigen could also be transferred by conjugation but its expression appeared to be controlled by genes at no less than two loci. Results of recombination experiments indicated that the potential for expressing the L antigen can be present without expression. In crosses in which the donor parent carried the latent ability to express the L antigen and the receptor parent carried an L antigen of another specificity, recombinants were obtained which had the same specificity as that of the donor parent. The mucoid property of recombinants obtained in K12 x B crosses by Calef and Cavalli (of Ørskov and Ørskov, 1960) also was thought to be controlled by a pair of complementary genes. Ørskov and Ørskov (1961) describe an F+ strain which, when converting other strains to the F+ state, also transfers the genetic determinant for the L antigen.

The fimbriate properties of E. coli B and of E. coli K12 have been studied extensively by Brinton (1959). Fimbriation in these strains was found to be correlated with colonial morphology but not with presence or absence of flagella nor with F status. Strain W677 F- was found to be fimbriated while 58-161 F+ was not. Fourteen of nineteen strains examined were found to have the potentiality for fimbriation. These strains demonstrated a phase variation between a fimbriated

and a non-fimbriated state. Reversions of the fimbriated state were found to occur at a frequency of about 4×10^{-4} per cell per generation. Crosses between a fimbriated K12 strain and a permanently non-fimbriated K12 mutant have revealed a chromosomal locus for a gene *fim*$^-$ at a site in the T L region (Maccacaro, *et al*, 1959). Data from these crosses suggested that expression of the *fim*$^+$ gene may depend in part on a cytoplasmic factor. The fimbriate property also has been transferred by conjugation from an Hfr strain of *E. coli* K12 to a non-fimbriated *Salmonella* species (Brinton and Baron, 1960).

The apparent low fertility of F+ derivatives of MSC501 obtained in the present investigation (see Table VI) deserves further study. Such differences in fertility observed elsewhere have been attributed to a mutant F+ factor (Clark and Adelberg, 1962) but this explanation seems unlikely in the present case since the source of F was identical for all three cultures. F+ and Hfr strains which have lost their ability to adsorb a male-specific bacteriophage have also been demonstrated to show a loss in degree of fertility (Clark and Adelberg, 1962).

The negative correlation of high motility with presence of *HSA*$^+$ in strain W6 (see section E) is precedented by reported instances in which the replication of cytoplasmic elements has been shown to be repressed in certain genetic backgrounds. An F factor can incorporate a marker from the bacterial chromosome and thus transfer this factor from cell to cell at high frequency. An F *lac*$^+$ factor, which transfers the

lac gene is unable to replicate normally in cells which contain, in addition, a normal sex factor. Replication of F *lac*$^+$ is completely inhibited in Hfr cells (Scaife and Gross, 1962). A similar influence is evidenced in the case of a colicin factor. The genetic basis of Colicin I, when present in strains of *E. coli* K12 is able to initiate its own transfer as well as the transfer of chromosomal markers in the absence of F. The kinetics of colicin factor transfer is influenced by the presence of F in either the *colI*$^+$ donor or the *colI*$^-$ recipient (Monk, 1962).

SUMMARY

The flocculent agglutination produced by many strains of *E. coli* K12 in response to antiserum is due in part to the presence of flagellar antigen and in part to a heat stable antigen (HSA) not associated with flagella.

Presence of HSA is not obviously correlated with F. status. Appropriate crosses show its genetic basis not to be closely linked to the TLB_1 region.

Occasional mutations from HSA^+ to HSA^- and the reverse have occurred simultaneously with mutations of other genes not obviously related physiologically.

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