Genetic Determination of the Antigenic Specificity of Flagellar Protein in *Salmonella*

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SUMMARY

Using non-flagellate (*fla*⁻) mutants of gn-complex antigenic derivatives of a stable phase-1 strain of *Salmonella abortusequi*, the positions of the antigenic specificity-determining sections on the genetic map of *H1*, a structural gene for flagellar protein, were located by establishing a system for selecting intra-*H1* recombinants. Before the recombination experiments, a factor analysis of the gn-complex antigens of the strains was made by cross-absorption tests: nine factors were detected.

P22 phage-mediated transductions were carried out between different pairs of *H1*-linked *fla*⁻ mutants, whose *H1*-alleles were different and whose *fla*⁻ sites were on opposite sides of *H1*. The *fla*⁺ transductants, present as swarms in semi-solid medium, were isolated and the composition of their flagellar antigens examined with specific antisera. Among 1366 *fla*⁺ transductants obtained, 26 clones were shown to have 'recombinant antigens' carrying some factors of one or both of the parental-type antigens. With several 'recombinant antigens', some new specificities with weak antigenicity were detected. The flagellar protein, 'flagellin', of an 'antigen recombinant' was proved to be a recombinant of the two parental flagellins by finger-printing analysis of tryptic digests.

On the assumption that these 'antigen recombinants' resulted from a single cross-over within *H1*, the relative positions of the antigenic specificity-determining sections within donor and recipient *H1* with regard to the cross-over point were obtained for individual 'antigen recombinants'. Summarizing the relative positions thus inferred, it was shown that each of the antigenic specificity-determining sections maps as a unit; and that as a whole they form a linear array within *H1*. Sections specifying some tryptic peptides of flagellins were also mapped within *H1*.

INTRODUCTION

Salmonella flagella exhibit a wide variety of antigenic specificities (Kauffmann, 1964). Many *Salmonella* species manifest two alternative types of flagellar antigen: one is called phase-1 and the other phase-2 (Andrews, 1922). Genetical analysis by transduction shows that the phase-1 and phase-2 specificities are determined by allelic genes at two loci, *H1* and *H2* (Lederberg & Edwards, 1953). Chemically, a *Salmonella* flagellum is a polymer of a single protein, flagellin, the molecular weight of which is 36,000 to 40,000 (Kerridge, Horne & Glauert, 1962; Asakura, Eguchi & Iino, 1964; McDonough, 1965). The genes *H1* and *H2* are therefore the structural genes specifying the primary structures of the flagellins of the respective phases (Iino & Lederberg, 1964).

Immunological tests show that the flagellar antigen is a complex composed of

several antigenic factors. That is, one flagellin molecule carries several distinct antigenic determinants. The so-called gn-complex antigens, a group of phase-I antigens possessing the factor g in common, have been analysed antigenically in detail, as shown in the Kauffman-White scheme (Kauffmann, 1964). After reviewing the compositions of naturally occurring gn-complex antigens, Iino (1959) proposed that the antigenic specificities are determined by several sections in the *H* gene, each corresponding to a different antigenic specificity. According to this hypothesis, intragenic recombination between different alleles of *H1* or *H2* will result in the production of a recombinant antigen carrying specificities derived from both parents, unless the specificity of each antigen is altered by conformational changes in the flagellin molecule resulting from recombination. Analysis of such recombinant antigens should therefore disclose the arrangement of antigen-determining sections within *H* genes. Some evidences of intragenic recombination between the *H2* alleles, *H2-1,2* and *H2-e,n,x* (Iino, 1960), and between some mutant alleles of *H1-i* (Joys & Stocker, 1963) has already been obtained, but the ordering of specificity-determining sections has not previously been attempted.

In the present study, an attempt has been made to map the antigenic specificity-determining sections in *H1* by intragenic recombination between different alleles of *H1-g*...

METHODS

Bacteria. *Salmonella dublin* NCTC4197 (1,9,12; g,p:-), *S. derby* NCTC1729 (1, 4, 5, 12; g,f:-), and *S. enteritidis* NCTC4196 (1,9,12; g,m:-) were received from Dr Joan Taylor (Central Public Health Laboratory, London). *Salmonella* sp. strain SJ6 (?; g,t:-) is a stock strain of this Institute. These naturally occurring strains are heterogeneous in various characters, for example, in their somatic antigens, phage sensitivity, etc. For the present study, four artificial strains were prepared by transferring the alleles of *H1-g*... by P22 phage-mediated transduction from the above four naturally occurring strains to a single recipient strain SJ241 (4, 12; a: (e,n,x)), a phase-I stable strain of *S. abortusequi*. Their strain numbers and some characteristics are shown in Table 1. The artificial strains are thought to have the same genetic background except *H1* and its vicinity. Since they are stable in phase-I and have identical somatic antigens, only phase-I antigens are indicated here when describing their antigenic formulae. All of them are *nml*⁺.

Non-flagellate (*fla*⁻) mutants were selected from strains TR6, TR16 and TR17 by phage M8, a mutant of phage χ . This phage attacks motile *Salmonella* organisms except for some specific serotypes including gn-complex antigenic *Salmonella*, but cannot attack non-flagellate (*fla*⁻) or paralysed (*mot*⁻) cells (Meynell, 1961). Phage M8 is a host-range mutant able to attack motile salmonellas with gn-complex antigens (Sasaki, 1962; Yamaguchi, 1968). The method used here for selecting *fla*⁻ mutants was that described by Joys & Stocker (1965) for isolating *fla*⁻ mutants from *S. typhimurium* LT2 with the χ -phage. The *fla*⁻ mutants used in the present study are listed in Table 2. They are stable and their mutation sites are very closely linked to *H1*. Complementation tests with the mutants of the already known *fla* cistrons showed that TR16-*fla*-227 and TR17-*fla*-251 carry deletions in the *flaA* cistron and that TR6-*fla*-373 has a deletion extending over the *flaA*, *B*, and *D* cistrons. The other three mutants, TR6-*fla*-351, TR16-*fla*-204, and TR17-*fla*-263, do not complement each other but com-

plement with every *fla*⁻ mutant of the known *fla* cistrons. The possibly new cistron represented by these three mutants is tentatively described as *flaL* in this paper. Reciprocal transductions were made between each of these mutants and a *fla*⁻ mutant of SJ241 (*flaA*⁻ *Hr-a*), and the flagellar antigen types of the *fla*⁺ transductants examined. From the ratios of the transductants carrying the donor-type antigen to those carrying the recipient-type antigen in every cross, it was shown that the *flaA* cistron and the *flaL* cistron are on the opposite sides of *Hr*. Details of the mapping of these *fla*⁻ sites will be published elsewhere.

Table 1. *Derivatives of Salmonella abortusequi SJ241, given Hr-g...-alleles from gn-complex antigenic Salmonella by transduction*

Strain no.	O antigens	H antigens		Sensitivity to phage		<i>nml</i> *	Donor of Hr-g...
		phase 1	phase 2	P22	M8		
TR6	4,12	g,t	(e,n,x)†	s‡	s	+	<i>Salmonella</i> sp. SJ6
TR11	4,12	g,p	(e,n,x)	s	s	+	<i>S. dublin</i> NCTC4197
TR16	4,12	f,g	(e,n,x)	s	s	+	<i>S. derby</i> NCTC1729
TR17	4,12	g,m	(e,n,x)	s	s	+	<i>S. enteritidis</i> NCTC4196

* The *nml* character was determined by the presence or absence of *ε*-N-methyllysine in acid-hydrolysed flagellin examined in the Hitachi automatic amino acid analyser.

†(): unexpressed antigen type.

‡ s: 'sensitive' to the indicated phage.

Table 2. *Properties of non-flagellate mutants of Salmonella*

Frequency of co-transduction with *Hr*: percentage of *fla*⁺ clones with the phase-1 antigen of donor in the total *fla*⁺ transductants produced in the P22 phage-mediated transduction from *Salmonella abortusequi* SJ241 (a: (e,n,x)) to the *fla*⁻ mutant. About 500 *fla*⁺ transductants were examined for each strain.

Complementation group: examined by trail formation in the P22 phage-mediated transduction from the *fla*⁻ mutant to standard mutants of the known *fla* cistrons derived from *S. typhimurium* TM2 (Iino & Enomoto, 1966). The sequence of the *fla* cistrons is *his...flaB-flaD-flaA-Hr-flaL...motA,B...trp*.

Strain	Reversion	Frequency of co-transduction with <i>Hr</i> (%)	Complementation group	Original strain
TR6- <i>fla</i> -351	—	89	L	TR6
TR6- <i>fla</i> -373	—	87	A, B, D	TR6
TR16- <i>fla</i> -204	—	96	L	TR16
TR16- <i>fla</i> -227	—	88	A	TR16
TR17- <i>fla</i> -251	—	54	A	TR17
TR17- <i>fla</i> -263	—	90	L	TR17

Media. Nutrient broth was composed of 1% (w/v) peptone and 1% (w/v) meat extract, adjusted to pH 7.2. Nutrient agar was prepared by the addition of 1.5% (w/v) agar to nutrient broth. Semi-solid medium (Edwards & Bruner, 1942; Stocker, Zinder & Lederberg, 1953) was prepared by adding 0.35% (w/v) agar and 8% (w/v) gelatin to the nutrient broth. All cultures were incubated at 37°.

Immunization procedure. Since the content of flagellar antigen of a bacterium is roughly proportional to its motility, the bacteria used for immunization, agglutination or absorption were first passed through semi-solid medium. Overnight broth cultures inoculated with highly motile organisms were centrifuged and the pellet suspended in

formalinized saline (formaldehyde concentration: 0.5%) to a final concentration of about 5×10^8 bacteria/ml. Rabbits were immunized by one intraperitoneal and five successive intravenous injections of a total of 6 ml. of bacterial suspension at 3-day intervals, and bled 1 week after the final injection. The antisera were processed according to Edwards & Ewing (1955).

Absorption of antisera. Highly motile organisms of an absorbing strain were grown in nutrient broth overnight, and used to inoculate trays ($20 \times 30 \times 1.8$ cm.) each containing 200 ml. nutrient agar. After incubation for 24 hr, the bacteria were harvested in formalinized saline (formaldehyde concentration: 0.5%), and the suspension kept at room temperature overnight. The bacteria were then centrifuged, resuspended in sterile saline and kept at 0°. This suspension was centrifuged before use and the pellet mixed with the antiserum to be absorbed in the proportion of about 0.5 g. wet weight organisms to 1.0 ml. serum. After 2 hr at 37° the mixture was centrifuged. If this once-absorbed serum still agglutinated the absorbing organisms, this process was repeated until no activity with the absorbing organisms remained.

Titration of antisera. The titres of antisera were determined by tube agglutination of two-fold serial dilutions incubated at 52° for 2 hr and expressed as the reciprocal or its logarithm (to base 2) of the highest dilution showing activity.

*Transduction between *fla*⁻ mutants.* Phage P 22 (Zinder & Lederberg, 1952) was used as transducing phage throughout. The phage was propagated by the soft agar-layer method (Adams, 1959) on a donor strain, and the lysate mixed with broth culture of the recipient (about 5×10^8 cells/ml.) at a m.o.i. of 5 to 10. After 10 min. at 37°, the mixture was diluted 10^{-1} and 10^{-2} with nutrient broth, and the dilutions brushed in line on semi-solid medium. The *fla*⁺ transductants were selected as swarms after incubation for 15 hr.

Preparation of flagellin. Broth cultures of highly motile bacteria were inoculated on nutrient agar in trays. After incubation for 24 hr at 37°, the bacteria were harvested in saline, washed, resuspended in distilled water, and de-flagellated by shaking (about 750 strokes/min., 25 mm. amplitude). The suspension was centrifuged at 3000 rev./min. for 40 min. The supernatant, containing the flagella, was then centrifuged at 30,000 rev./min. in a Spinco Model L ultracentrifuge for 45 min. The deposited flagella were disintegrated into monomers of flagellin by treatment with 0.01 N-HCl for 30 min. at room temperature. The solution was then centrifuged at 30,000 rev./min. for 45 min. to remove turbidity, and the supernatant dialysed for 5 hr against three changes of distilled water and lyophilized *in vacuo*. No further preparation was undertaken since the flagellin preparation thus obtained showed only one peak and one band in column chromatography using DEAE-cellulose and electrophoresis, respectively (Enomoto & Iino, 1962).

Tryptic peptide maps. Lyophilized flagellin was dissolved in 0.05 M-phosphate buffer (pH 8.0) at 2%, denatured by heating at 90° for 5 min., and digested with trypsin (2%) at 25° for 24 hr. A sample of the digest was applied to a sheet (40×30 cm.) of Tōyō no. 50 filter paper and subjected to electrophoresis in pyridine + acetic acid + water (135 + 15 + 1 by vol.) at pH 6.65 for 3.5 hr at 15 V/cm. After drying, the paper was subjected twice to ascending chromatography with *n*-butyl alcohol + acetic acid + water (3 + 1 + 1 by vol.) and the peptide spots developed with ninhydrin dissolved in acetone (0.25 %, w/v).

RESULTS

Further analysis of factor compositions of gn-complex antigens

Although the compositions of the gn-complex antigens have been analysed in some detail, it has often been recognized that the factor g may be further subdivided into several factors (e.g. Edwards & Ewing, 1955). Thus, an analysis of the gn-complex antigens was carried out with the four gn-complex antigenic strains, TR6 (g,t), TRII (g,p), TRI6 (f,g), TRI7 (g,m), by cross-absorption.

Table 3. *Cross-absorption-agglutination test of anti-gn-sera*

Antiserum	Absorbed with	Activity of absorbed antiserum on			
		TR6	TRII	TRI6	TRI7
Anti-TR6	TRII	+	—	+	+
	TRI6	+	+	—	+
	TRI7	+	—	+	—
	TRII and TRI6	+	—	—	+
	TRII and TRI7	+	—	+	—
	TRI6 and TRI7	+	—	—	—
Anti-TRII	TR6	—	+	+	+
	TRI6	+	+	—	+
	TRI7	—	+	—	—
	TR6 and TRI6	—	+	—	+
	TR6 and TRI7	—	+	—	—
	TRI6 and TRI7	—	+	—	—
Anti-TRI6	TR6	—	+	+	+
	TRII	+	—	+	—
	TRI7	+	—	+	—
	TR6 and TRII	—	—	+	—
	TR6 and TRI7	—	—	+	—
	TRII and TRI7	+	—	+	—
Anti-TRI7	TR6	—	+	+	+
	TRII	+	—	—	+
	TRI6	+	+	—	+
	TR6 and TRII	—	—	—	+
	TR6 and TRI6	—	+	—	+
	TRII and TRI6	+	—	—	+

+, Agglutination occurred; —, no agglutination occurred.

Several rabbits were immunized with TR6, TRII, TRI6 and TRI7, respectively. Anti-sera of titre of 12,800 to 25,600 against the homologous antigens were obtained. These were fully absorbed with organisms of one or two non-homologous test strains, and the residual cross-reactivities examined by slide agglutination (Table 3). Since the somatic (o) antigens are common to all the strains used, the activities remaining after absorption are those for the factor(s) present on the flagellar antigen of the immunizing strain but absent from that of the absorbing strain. The results indicated the presence of at least nine factors in the gn-complex antigens examined. Four were specific to the respective strains and were therefore regarded, respectively, as factors t, p, f, and m in the Kauffmann-White scheme. The other five factors common to two or three strains were regarded as parts of a complex of factors, which has so far been given the symbol g; they will be described as g₁, g₂, g₃, g₄ and g₅, respectively. The factor

compositions of the four strains are shown in Table 4. There remained the possibility that besides these nine factors a factor or factors common to all the four antigens exists, which would have been overlooked in the present cross-absorption test.

Table 4. *Factors present in the gn-complex antigens*

(g₀): a factor or a complex of factors common to all these gn-complex antigens which would not be detected in the present cross-absorption test.

Strain	Factor composition								
	(g ₀)	g ₁	g ₂	g ₃	.	.	t	.	.
TR6	(g ₀)	g ₁	g ₂	g ₃	.	.	t	.	.
TR11	(g ₀)	g ₁	.	.	g ₄	g ₅	.	p	.
TR16	(g ₀)	.	.	g ₃	g ₄	.	.	.	f
TR17	(g ₀)	g ₁	g ₂	.	g ₄	g ₅	.	.	m

Table 5. *Anti-factor sera used for detecting the factors of flagellar antigens of fla⁺ recombinants from different pairs of fla⁻ mutants*

Factor to be detected	Anti-factor(s) serum used	Preparation of the anti-factor(s) serum	
		Serum	Absorbed with
(a) For <i>fla</i> ⁺ transductants between <i>fla</i> ⁻ mutants of TR6 and TR16			
<i>g</i> ₁	Anti- <i>g</i> ₁ , <i>g</i> ₅ , <i>p</i>	Anti-TR11	TR16
<i>g</i> ₂	Anti- <i>g</i> ₂ , <i>m</i>	Anti-TR17	TR11
<i>g</i> ₄	Anti- <i>g</i> ₄ , <i>g</i> ₅ , <i>p</i>	Anti-TR11	TR6
<i>t</i>	Anti- <i>t</i>	Anti-TR6	TR16 and TR17
<i>f</i>	Anti- <i>f</i>	Anti-TR16	TR6 and TR11
(b) For <i>fla</i> ⁺ transductants between <i>fla</i> ⁻ mutants of TR6 and TR17			
<i>g</i> ₃	Anti- <i>g</i> ₃ , <i>f</i>	Anti-TR16	TR17
<i>g</i> ₄	Anti- <i>g</i> ₄ , <i>f</i>	Anti-TR16	TR6
<i>g</i> ₅	Anti- <i>g</i> ₅ , <i>p</i>	Anti-TR11	TR6 and TR16
<i>t</i>	Anti- <i>t</i>	Anti-TR6	TR16 and TR17
<i>m</i>	Anti- <i>m</i>	Anti-TR17	TR6 and TR11
(c) For <i>fla</i> ⁺ transductants between <i>fla</i> ⁻ mutants of TR16 and TR17			
<i>g</i> ₁	Anti- <i>g</i> ₁ , <i>g</i> ₅ , <i>p</i>	Anti-TR11	TR16
	Or anti- <i>g</i> ₁ , <i>g</i> ₂ , <i>t</i>	Anti-TR6	TR16
<i>g</i> ₂	Anti- <i>g</i> ₂ , <i>t</i>	Anti-TR6	TR11 and TR16
<i>g</i> ₃	Anti- <i>g</i> ₃ , <i>t</i>	Anti-TR6	TR17
<i>g</i> ₅	Anti- <i>g</i> ₅ , <i>p</i>	Anti-TR11	TR6 and TR16
<i>f</i>	Anti- <i>f</i>	Anti-TR16	TR6 and TR17
<i>m</i>	Anti- <i>m</i>	Anti-TR17	TR6 and TR11

Recombination within *H1*

The system for detecting recombinants was as follows. P22 phage-mediated transduction was done between a pair of non-flagellate (*fla*⁻) mutants whose *H1*-alleles differed; whose *fla*⁻ sites were cotransduced with *H1* in high frequency; and were on the opposite sides of *H1*: that is, between *fla*⁻ mutants *fla*-a (-*fla*-a⁻ - *H1*-A - *fla*-b⁺-) and *fla*-b (-*fla*-a⁺ - *H1*-B - *fla*-b⁻-). The *fla*⁺ transductants developing as swarms in semi-solid medium were isolated and their antigenic compositions examined. The occurrence of an *fla*⁺ transductant in this case required at least one cross-over in the region between the two *fla*⁻ sites surrounding *H1*. Thus, the closer the *fla*⁻ sites to *H1*, the larger should be the relative frequency of intra-*H1* recombinants among the *fla*⁺ transductants.

Table 6. Factors present in the flagellar antigens of *fla*⁺ recombinants from different pairs of *fla*⁻ mutants

Factor composition of flagellar antigen: examined by slide agglutination with anti-factor sera listed in Table 5.

A, B, D and L in parentheses: complementation groups of *fla*⁻ mutants.

Transduction		<i>fla</i> ⁺ transductant						Strain no. of 'antigen type recombinant'			
Donor	Recipient	No. of clones	Factor composition of flagellar antigen								
(a) The <i>fla</i> ⁺ transductants between TR6- and TR16- <i>fla</i> ⁻ mutants											
			<i>g</i> ₁	<i>g</i> ₂	<i>g</i> ₄	<i>f</i>	<i>t</i>				
TR6- <i>fla</i> -373 (A,B,D)	TR16- <i>fla</i> -204 (L)	63	+	+	-	-	+	(TR6-type)	SJ1740 to SJ1742 SJ1743		
		32	-	-	+	+	-	(TR16-type)			
		3	-	-	+	-	+	(Rec.-type I)			
		1	-	-	-	-	+	(Rec.-type II)			
TR16- <i>fla</i> -204 (L)	TR6- <i>fla</i> -373 (A,B,D)	85	+	+	-	-	+	(TR6-type)	.		
		15	-	-	+	+	-	(TR16-type)		.	
TR6- <i>fla</i> -351 (L)	TR16- <i>fla</i> -227 (A)	28	+	+	-	-	+	(TR6-type)	.		
		68	-	-	+	+	-	(TR16-type)		.	
		3	+	+	+	+	-	(Rec.-type III)		SJ1744 to SJ1746	
TR16- <i>fla</i> -227 (A)	TR6- <i>fla</i> -351 (L)	35	+	+	-	-	+	(TR6-type)	.		
		64	-	-	+	+	-	(TR16-type)		.	
		1	-	+	+	+	-	(Rec.-type IV)		SJ1747	
(b) The <i>fla</i> ⁺ transductants between TR6- and TR17- <i>fla</i> ⁻ mutants											
			<i>g</i> ₃	<i>g</i> ₄	<i>g</i> ₅	<i>m</i>	<i>t</i>				
TR6- <i>fla</i> -373 (A,B,D)	TR17- <i>fla</i> -263 (L)	56	+	-	-	-	+	(TR6-type)	.		
		44	-	+	+	+	-	(TR17-type)		.	
TR17- <i>fla</i> -263 (L)	TR6- <i>fla</i> -373 (A,B,D)	88	+	-	-	-	+	(TR6-type)	.		
		52	-	+	+	+	-	(TR17-type)		.	
TR6- <i>fla</i> -351 (L)	TR17- <i>fla</i> -251 (A)	17	+	-	-	-	+	(TR6-type)	.		
		91	-	+	+	+	-	(TR17-type)		.	
TR17- <i>fla</i> -251 (A)	TR6- <i>fla</i> -351 (L)	23	+	-	-	-	+	(TR6-type)	.		
		98	-	+	+	+	-	(TR17-type)		.	
		1	-	-	+	+	+	(Rec.-type V)		SJ1748	
(c) The <i>fla</i> ⁺ transductants between TR16- and TR17- <i>fla</i> ⁻ mutants											
			<i>g</i> ₁	<i>g</i> ₂	<i>g</i> ₃	<i>g</i> ₅	<i>f</i>	<i>m</i>			
TR16- <i>fla</i> -227 (A)	TR17- <i>fla</i> -263 (L)	74	-	-	+	-	+	-	(TR16-type)	.	
		56	+	+	+	+	-	+	(TR17-type)		.
		7	+	+	+	-	+	-	(Rec.-type VI)		SJ1749 to SJ1755
TR17- <i>fla</i> -263 (L)	TR16- <i>fla</i> -227 (A)	92	-	-	+	-	+	-	(TR16-type)	.	
		35	+	+	+	+	-	+	(TR17-type)		.
		5	+	+	+	-	+	-	(Rec.-type VII)		SJ1756 to SJ1760
TR16- <i>fla</i> -204 (L)	TR17- <i>fla</i> -251 (A)	1	-	+	+	-	+	-	(Rec.-type VIII)	SJ1761	
		11	-	-	+	-	+	-	(TR16-type)		.
		71	+	+	+	+	-	+	(TR17-type)		.
		2	-	-	-	+	-	+	(Rec.-type IX)		SJ1762 and SJ1763
TR17- <i>fla</i> -251 (A)	TR16- <i>fla</i> -204 (L)	22	-	-	+	-	+	-	(TR16-type)	.	
		120	+	+	+	+	-	+	(TR17-type)		.
		1	-	-	-	+	-	+	(Rec.-type X)		SJ1764
		1	+	-	-	+	-	+	(Rec.-type XI)		SJ1765

Transductions were made in the combinations mentioned above, using two *HI*-linked *fla*⁻ mutants each of TR6, TR16 and TR17, listed in Table 2; the *fla*⁻ sites of the two were on the opposite sides of *HI*. The antigenic compositions of the *fla*⁺ transductants obtained were examined by slide agglutination tests with anti-factor(s) sera (Table 5). Since not all the antisera for single factors were available, antisera for a complex of factors were sometimes used. For example, anti-*g*₁, *g*₅, *p* serum was used to detect factor *g*₁ of the transductants formed in the cross between *fla*⁻ mutants of TR6 (*g*₁, *g*₂, *g*₃, *t*) and TR16 (*g*₃, *g*₄, *f*), because factors *g*₅ and *p* were absent from both of the parental type antigens. As another example, anti-*g*₁, *g*₅, *p* and anti-*g*₁, *g*₂, *t* sera were used to detect factor *g*₁ of transductants obtained from crosses between *fla*⁻ mutants of TR16 (*g*₃, *g*₄, *f*) and TR17 (*g*₁, *g*₂, *g*₄, *g*₅, *m*). The former was used for the detection of *g*₁ in clones lacking factor *g*₅, and the latter for *g*₁ in those lacking factor *g*₂.

The majority (1340 clones) of the total 1366 *fla*⁺ transductants had either donor- or recipient-type antigens, while the remaining 26 had new combinations of antigens (Table 6). For example, four types of transductant clones were obtained in transducing from TR6-*fla*-373 (*HI-g*₁, *g*₂, *g*₃, *t*) to TR16-*fla*-204 (*HI-g*₃, *g*₄, *f*) as shown in Table 6 (a). Among them, the first and second were the donor- and the recipient-types, respectively, and the remaining two were new types; the one carried factor *t* of the donor together with *g*₄ of the recipient, and the other carried only *t* of the donor, among the factors tested. All in all, eleven such new types (recombinant-type I to XI in Table 6) were found in the present experiment. Among them, seven types had some factors of both of the donor- and the recipient-type antigens, and the other four had only part of the factors of one of the parental type antigen. On the supposition that these new type clones arose by intragenic recombination between alleles of *HI-g*..., they were designated as 'antigen recombinants', and the new type antigens as 'recombinant antigens.'

Serological nature of 'recombinant antigens'

One strain of each of the eleven types of recombinant was examined for the antigenic reactivity of their individual factors and also for the existence of antigenic factors absent from the parental type antigens. Individual factors were examined by measuring the titres of anti-factor(s) sera with organisms of the test strains. These sera were those used to detect 'antigen recombinants' (Table 5). As shown in Table 7, the reactivity of individual factors of the 'recombinant antigens' was almost equal to that of the corresponding factors of the parental type antigens.

A rabbit was immunized with actively motile cells of each 'antigen recombinant' to be tested. This antiserum was then repeatedly absorbed with a mixture of the parental strains. Since the 'antigen recombinants' used as immunogens had been lysogenized with P22 phage, which is known to cause formation of somatic antigen 1 by the host organism (Zinder, 1957), the absorbing bacteria were first lysogenized with the phage. After every absorption, residual activity was titrated with the homologous antigen by tube agglutination (Table 8). Antisera, except those against SJ1740, SJ1747 and SJ1749, were easily inactivated by several absorptions; that is, there was no factor on these 'recombinant antigens' other than those present on the parental type antigens. Weak activities remained against SJ1740, SJ1747 and SJ1749 after full absorption. These fully absorbed antisera did not agglutinate any antigen used here other than their respective

homologous antigens. Also they did not agglutinate salmonellas with the following flagellar antigens: a; b; e, n, x; i; 1, 2; g, m, s; m, t; g, m, q; g, s, t; g, q.

Although some 'recombinant antigens' had a unique antigenic pattern, the results obtained here indicate that of the 'recombinant antigens', at least seven types were almost complete mosaics, made up partly of donor-type antigen and partly of recipient-type antigen. The other six types may also be regarded as molecular mosaics, assuming that an active part of the antigen of one parent antigen had been replaced by an inactive part from the other parent.

Table 7. *Antigenic reactivity of individual factors of the flagellar antigens in the 'antigen recombinants'*

Titres of the anti-factor(s) antisera on the indicated antigens were determined by tube agglutination and expressed as log to base 2.

Antigen		Log titre to base 2 of anti-factor(s) serum (Factor to be examined)						
(a) 'Antigen type recombinants' between TR6- and TR16- <i>fla</i> ⁻ mutants								
		Anti-g ₁ ,g ₆ ,p (g ₁)	Anti-g ₂ ,m (g ₂)	Anti-g ₄ ,g ₅ ,p (g ₄)	Anti-f (f)	Anti-t (t)		
TR6	} (Parental type)	5	7	< 0	< 0	11		
TR16		< 0	< 0	9	9	< 0		
SJ1740	(Rec.-type I)	< 0	< 0	7	< 0	11		
SJ1743	(Rec.-type II)	< 0	< 0	< 0	< 0	11		
SJ1744	(Rec.-type III)	5	7	8	9	< 0		
SJ1747	(Rec.-type IV)	< 0	7	9	8	< 0		
(b) 'Antigen type recombinants' between TR6- and TR17- <i>fla</i> ⁻ mutants								
		Anti-g ₃ ,f (g ₃)	Anti-g ₄ ,f (g ₄)	Anti-g ₅ ,p (g ₅)	Anti-m (m)	Anti-t (t)		
TR6	} (Parental type)	7	< 0	< 0	< 0	13		
TR17		< 0	6	8	9	< 0		
SJ1748	(Rec.-type V)	< 0	< 0	7	9	13		
(c) 'Antigen type recombinants' between TR16- and TR17- <i>fla</i> ⁻ mutants								
		Anti-g ₁ ,g ₆ ,p (g ₁)	Anti-g ₁ , g ₂ ,t(g ₁)	Anti-g ₂ ,t (g ₂)	Anti-g ₃ ,t (g ₃)	Anti-g ₅ ,p (g ₅)	Anti-f (f)	Anti-m (m)
TR16	} (Parental type)	< 0	< 0	< 0	10	< 0	10	< 0
TR17		nt	nt	10	< 0	8	< 0	10
SJ1749	(Rec.-type VI)	4	nt	10	9	< 0	9	< 0
SJ1756	(Rec.-type VII)	5	nt	10	10	< 0	9	< 0
SJ1761	(Rec.-type VIII)	< 0	nt	9	10	< 0	10	< 0
SJ1762	(Rec.-type IX)	nt	< 0	< 0	< 0	8	< 0	10
SJ1764	(Rec.-type X)	nt	< 0	< 0	< 0	8	< 0	10
SJ1765	(Rec.-type XI)	nt	5	< 0	< 0	8	< 0	10

nt = not tested

Tryptic peptide maps of flagellins of 'antigen recombinants' and parental-type strains

To show that the 'antigen recombinants' had resulted from recombination within *Hr*, flagellins were isolated from the parental strains and from one strain of each of the eleven types of 'antigen recombinants', and their tryptic digests analysed by the finger-printing technique.

Fig. 1. Tracing of the tryptic peptide maps of flagellins of TR6, TR16 and TR17, gn-complex antigenic derivatives of a phase-I stable strain of *Salmonella abortusequi*, and SJ1740 and SJ1744, 'antigen recombinants' between TR6 and TR16. Electrophoresis at pH 6.7 followed by twice of ascending chromatography with *N*-butanol+acetic acid+water (3+1+1).

Table 8. Log titres (to base 2) of antisera against 'recombinant antigens' after every absorption with their parental type antigens

Antiserum was absorbed with the mixture of 0.5 g (in wet weight) of each absorbing strains per 1.0 ml. serum at one time. Organisms lysogenized with P 22 phage were used for absorption. After every absorption, the remaining activity was tested on the homologous antigens by tube agglutination and expressed as log titre to base 2.

Serum	Absorbed with	Time of absorption				
		0	1	2	3	4
Anti-SJ1740 (Rec.-type I)	TR6 and TR16	15	8	4	3	3
Anti-SJ1743 (Rec.-type II)	TR6 and TR16	14	4	1	< 0	.
Anti-SJ1744 (Rec.-type III)	TR6 and TR16	14	4	1	< 0	.
Anti-SJ1747 (Rec.-type IV)	TR6 and TR16	15	9	5	4	4
Anti-SJ1748 (Rec.-type V)	TR6 and TR17	14	5	1	< 0	.
Anti-SJ1749 (Rec.-type VI)	TR16 and TR17	15	7	3	2	2
Anti-SJ1756 (Rec.-type VII)	TR16 and TR17	14	7	1	< 0	.
Anti-SJ1761 (Rec.-type VIII)	TR16 and TR17	15	7	1	< 0	.
Anti-SJ1762 (Rec.-type IX)	TR16 and TR17	14	5	1	< 0	.
Anti-SJ1764 (Rec.-type X)	TR16 and TR17	15	6	1	< 0	.
Anti-SJ1765 (Rec.-type XI)	TR16 and TR17	15	6	1	< 0	.

Tryptic peptide maps of flagellins of the three parental type strains and two of the 'antigen recombinants' are shown in Fig. 1. The approximate number of spots was 35 to 36 for every specimen and almost all the spots, except a few, seemed to be common to all parental and recombinant-type strains. Five spots in all were distinguishable among the maps of the parental-type strains: two spots (A, B in Fig. 1) were specific for TR6, two (C, D) for TR16, and one (E) for TR17. These different spots

were found in various combinations on the maps of the 'antigen recombinants', and no other spot except those found on the maps of the parental-type strains was detected. For example, three spots were found besides the common spots on the map of SJ1744, a recombinant of TR6 and TR16: one was the same as B specific for TR6 and the other two were the same as C and D specific for TR16, respectively. This proves that flagellin of SJ1744 was a mosaic, made up of parts of the flagellins of TR6 and TR16. The distribution of the spots A-E among the maps of the 'antigen recombinants' is shown in Table 9. It must be kept in mind that there must have been other different peptides which were indistinguishable in the finger-printing maps of the present experiment.

Table 9. *Distribution of the differing spots on the peptide maps of flagellins of the 'antigen recombinants'*

Recombinant between <i>fla</i> ⁻ mutants of	Strain	Differing spots*				
		A	B	C	D	E
(Parental type)	TR6	+	+	.	.	.
	TR16	.	.	+	+	.
	TR17	.	.	.	.	+
TR6 and TR16	SJ1740	+	.	.	.	.
	SJ1743	+	+	.	.	.
	SJ1744	.	+	+	+	.
	SJ1747	.	.	+	+	.
TR6 and TR17	SJ1748	+	+	.	.	.
TR16 and TR17	SJ1749	.	.	.	.	+
	SJ1756	.	.	+	+	.
	SJ1761	.	.	+	+	.
	SJ1762	.	.	.	.	+
	SJ1764	.	.	.	.	+
	SJ1765	.	.	.	.	+

* As for the differing spots A to E, refer to Fig. 1.

Mapping of antigenic specificity-determining sections and sections corresponding to some tryptic peptides of flagellins within H1

Provided that every 'antigen recombinant' resulted from a single cross-over within *H1*, their origins were inferred from their antigenic factor compositions (Table 6) and their tryptic peptide compositions (Table 9). The results are illustrated in Fig. 2 where *flaA* and *flaL* are placed on the left and right of *H1*, respectively. The italicized symbols *g*₁, *g*₂, *A*, etc. in the figure represent sections corresponding to the respective factors or peptides. From these inferred origins, an attempt was made to map antigenic specificity-determining sections and sections for the differing peptides within *H1*.

First, the 'antigen recombinants' between the *fla*⁻ mutants of TR6 and TR16 illustrated in Fig. 2 (*a*) will be considered. In SJ1747, *g*₂ is on the left and separated by the presumed cross-over point from the other sections *g*₁, *g*₄, *f*, *t*, *A*, *B*, *C* and *D*. In SJ1743, not only *g*₂ but also *g*₁ is separated from the other sections. Furthermore, *g*₂, *g*₁ and *B*, and *g*₂, *g*₁, *B*, and *g*₄ are separated from the other groups in SJ1744 and SJ1740, respectively. These suggest the following arrangement of the sections.

$$\begin{array}{c}
 \text{H1} \\
 \text{--- } \textit{flaA} - \overbrace{g_2 - g_1 - B - g_4 - (f, t, A, C, D)}^{\text{H1}} - \textit{flaL} -
 \end{array}$$

Fig. 2. Inferred origins of the 'antigen recombinants' through a single intra-*HI* recombination in P22 phage-mediated transduction. (a) Recombinants between *fla*⁻ mutants of TR6 and TR16; (b) between those of TR6 and TR17, and (c) between those of TR16 and TR17. D, donor; R, recipient. g₁, g₂, etc., antigenic specificity-determining sections. A, B, etc., sections in *HI* for the peptides A to E of the tryptic digests of flagellins (refer to Fig. 1).

Furthermore, SJ1748 (Fig. 2b), the only one 'antigen recombinant' obtained between *fla*⁻ mutants of TR6 and TR17, suggests the arrangement

$$\begin{array}{c} HI \\ \hline -flaA - (g_4, t, A, B, E) - (g_3, g_5, m) - flaL - \end{array}$$

In SJ1761 and SJ1765, the 'antigen recombinants' between *fla*⁻ mutants of TR16 and TR17 (Fig. 2c), *g*₂ is on the left separated from the other sections *g*₁, *g*₃, *g*₅, *f*, *m*, *C*, *D*, and *E*; and in SJ1715, SJ1762 and SJ1764, *g*₂ and *g*₁ are separated from others to the left. In SJ1749, *g*₂, *g*₁, *C*, *D*, and *E* are separated to the left. Thus, the following order is obtained:

$$\begin{array}{c} HI \\ \hline -flaA - g_2 - g_1 - (C, D, E) - (g_3, g_5, f, m) - flaL - \end{array}$$

On considering the results as a whole the order is that shown in Fig. 3. No contradiction has been found for any of the 'antigen recombinants' considered.

Fig. 3. Arrangement of the antigenic specificity-determining sections and locations of the sections for some tryptic peptides of flagellins within *HI*. Section *f* is somewhere to the right of *g*₄, *C*, *D*, and *E*. The orders of the sections in parentheses are not yet determined. *A*, *B*, *C*, *D*, and *E* are somewhere on the respective regions shown by dotted lines.

An attempt to assign antigenic specificities to the specific peptides

It is to be expected that a section specifying a tryptic peptide covering the whole or a part of an antigenic determinant of flagellin will behave in intra-*HI* recombination always in company with the antigenic specificity-determining section corresponding to that determinant. From this point of view, the sections *A* to *E* were examined for their association with any one of the antigenic specificity-determining sections by checking the data presented in Fig. 2. The section *A* was always associated with section *t* so far as the present study shows. The other sections *B*, *C*, *D*, and *E* were not associated with any one of the antigenic specificity-determining sections considered here.

DISCUSSION

Salmonella mutants with altered flagellar antigens may arise through mutation in the *H* gene (Iino & Mitani, 1964; Joys & Stocker, 1966; Yamaguchi & Iino, 1966). On occasion the serological character of some flagellar antigens is also modified by a gene, *nml* (Dr B. A. D. Stocker, personal communication), whose product, probably

an enzyme, is thought to methylate some lysine residues of flagellin to ϵ -*N*-methyllysine (NML) residues (Stocker, McDonough & Ambler, 1961; Kerridge, 1966). We must therefore consider the possibility of mutation in either *H1* or *nml* as well as of recombination within *H1* as the origin of the 'antigen recombinants' described here. Against this is the behaviour of mutants with altered flagellar antigens selected by cultivation of cells in semi-solid medium containing anti-flagellar antiserum. In this way, we isolated sixteen antigen type mutants from a gn-complex antigenic strain (Yamaguchi & Iino, 1966). The frequency of their occurrence was 10^{-6} to 10^{-7} /inoculated organism. In each case, a factor or several factors had been only slightly altered, so retaining cross-reactivity with the original factor or factors. A mutant that had lost one or more factors and/or had gained one or more rather strong ones, such as the 'antigen recombinants', was not found. The possibility may therefore be excluded that the 'antigen recombinants' occurring here at the high frequency of 2×10^{-2} (26 clones/1366 *fla*⁺ transductants) had arisen through mutation.

On the assumption that each 'antigen recombinant' arose by a single cross-over within *H1*, mapping within *H1* was performed. All the serological data and the results of finger-printing so far obtained are consistent with this assumption, indicating that each of the antigenic specificity-determining sections locates as a unit and that as a whole they are arranged linearly within *H1*.

The present study was made possible by the following facts: (1) *fla*⁻ mutants whose mutations are closely linked to *H1* and at either side of it were obtained; (2) as the result of further analysis of the factor compositions of the antigens used, many kinds of anti-factor(s) sera became available; (3) antigenic factors on the 'recombinant antigens' retained almost the same serological character as that of the corresponding factors on the parental type antigens.

In the Kauffmann-White scheme (Kauffman, 1964), nine factors of the gn-complex antigens were described: f, g, m, p, q, s, t, u, and z₅₁. In the present study, it has been shown that the factor g is a complex composed of two or more of at least 5 factors, g₁ to g₅. It must be mentioned here that, if these gn-complex antigens are modified by the presence or absence of ϵ -*N*-methyllysine (NML), namely *nml*⁺ or *nml*⁻, the analysis of the gn-complex antigens without NML may give somewhat differing results from the present one.

The presence of chromosomal sections marked with the respective antigenic specificities of flagellin within *H1* has been shown in the present study, but this does not necessarily mean that the specificity of an antigenic determinant of flagellin depends exclusively on the amino acid sequence or the primary structure specified by the corresponding section. It has been reported that ribonuclease oxidized by performic acid, a process which markedly alters its conformation without any change in its primary structure, does not cross-react serologically with native ribonuclease (Brown, 1962). It has also been reported that in *Salmonella*, several antigenic factors present on the wild-type flagellin may change simultaneously as a result of only one presumptive amino acid replacement (Joys & Stocker, 1966; Yamaguchi & Iino, 1966). Therefore, the amino acid sequence determined by an antigenic specificity-determining section manifests the specific antigenicity only when it has a specific tertiary structure in the whole conformation of the flagellin molecule.

Although some new specificities with weak antigenicity were detected on several 'recombinant antigens', all the 'recombinant antigens' obtained here are almost

perfect mosaics of the parental antigens in their serological nature. This implies that the tertiary structure of individual antigenic determinants on the recombinant flagellins had suffered little or no change by recombination. This may also be expected from the strong resemblance between the tryptic peptide maps of the parental flagellins. Experiments to obtain 'recombinant antigens' between the non-cross-reacting antigens a and gn-complex were carried out in parallel with the present study, but were unsuccessful. One of the reasons may be that since a and gn-complex flagellins differ considerably in their amino acid composition (McDonough, 1965), recombinant flagellins, if produced, might be so abnormal in their conformation that they could not form motile flagella.

In globular proteins like flagellin, the folding of the polypeptide chains brings into apposition amino acids remote from each other in the primary structure. Some antigenic determinants may therefore include amino acids from two or more points along the chain. For such a determinant, the antigenic specificity-determining section should locate as two or more units within the structural gene. Although it was shown that each of the antigenic specificity-determining sections considered in the present study is located as a unit within *H1*, this does not exclude the above possibility.

Many attempts have been made to define the structural features of antigenic determinants of globular proteins by isolating and characterizing fragments of the whole molecule which retain the ability to combine with specific antiserum. In the present study, an attempt was made to assign antigenic specificities of flagellin to some specific tryptic peptides, on the assumption that the tryptic peptide corresponding to an antigenic determinant on the flagellin molecule always corresponds to the antigenic specificity manifested by the determinant in intra-*H1* recombination. Though the data are as yet insufficient for complete assignment, the present results are sufficient to suggest that more peptide analysis will make this approach possible.

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