Phase Variation of Flagellar Antigens in *Salmonella*: Abortive Transduction Studies

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SUMMARY

Motility was transduced by phage P22 to non-motile (*fla*⁻, etc.) *Salmonella typhimurium* strains, and the flagellar antigens of abortive transductants inferred from the inhibition by antisera of the trails they produced in semi-solid medium. When the recipient had a *fla*⁻ gene closely linked to *H1* (phase-1 flagellar antigen locus) nearly all the *fla*⁺ abortive transductants evoked from a recipient culture in latent phase 1 manifested the phase-1 antigens of both recipient and donor, whereas those from a culture in latent phase 2 showed neither phase-1 antigen. Thus the expression of an exogenote *H1* allele, like that of the chromosomal *H1* allele, was regulated by the phase-determinant of the recipient, at or near *H2* (phase-2 flagellar antigen locus). An *H1* gene adjacent (*cis*) to *ahI*⁻ (*H1* activator gene), in the chromosome or in the exogenote, was unexpressed in *ahI*⁺ or *fla*⁺ abortive transductants in phase 1. This suggests that *ahI*⁻ mutants are *H1*-operator-negative mutants.

When the recipient was a phase-1, and therefore non-motile, culture of an *ahI*⁻ or 'phase-1-curly' mutant, lysates of phase-2 cultures, but not of phase-1 cultures of the same donor, evoked trails, attributable to *H2* abortive transductants. They expressed the donor *H2* allele, but not the recipient *H2* allele—nor the previously expressed *H1* allele of the 'phase-1-curly' recipient. It is inferred that a phase-determinant regulates the expression of the *H2* gene adjacent (*cis*) to it (or of which it forms part) but not that of another *H2* gene in the same cell; and that it controls the expression of *H1* via a repressor substance, not via an inducer. The exceptional *H1* allele *H1-1,2* determines a flagellin of antigenic character 1,2, apparently identical with that determined by a common *H2* allele. *H1-1,2* and the common *H1* allele *H1-b* were simultaneously expressed in *fla*⁺ abortive transductants, which suggests that neither *H2* flagellin nor *H2* messenger RNA functions as the repressor of *H1*. It is proposed that an operon, comprising *H2* and the structural gene for an *H1* repressor substance, has alternative metastable states, 'on' in phase 2 and 'off' in phase 1, comparable to the wild-type state and to that of an operator-negative mutant.

INTRODUCTION

Most *Salmonella* species have two flagellar antigens, the phase-1 and phase-2 antigens, and show diphasic variation. A given bacterium manifests only one of these two antigens (and is said to be in the corresponding phase) and produces descendants most of which are in the same phase as itself, but amongst these a minority in the

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other phase arise by an apparently random mutation-like process. In *S. typhimurium* and *S. abony* the rate of change from phase 1 to phase 2 ranges from about 10^{-5} to 10^{-3} per bacterium per generation, and of the reverse change from about 10^{-4} to 5×10^{-3} (Stocker, 1949; Mäkelä, 1964). The various phase-1 and phase-2 antigens which occur in different species or serotypes are determined by a series of alleles at two loci termed, respectively, *H1* and *H2*, which are widely separated on the bacterial chromosome (Smith & Stocker, 1962; Sanderson & Demerec, 1965; Mäkelä, 1964). Probably *H1* and *H2* are the structural genes for the phase-1 and phase-2 flagellar proteins (flagellins) which constitute the flagella of phase-1 or phase-2 bacteria (McDonough, 1965). The production of flagella of either antigenic type is prevented by *fla* mutations, some of which are closely linked to, and co-transducible with, *H1*. Though *fla*⁻ strains lack flagella, they undergo phase-variation, their (latent) flagellar antigenic phase being revealed by, for instance, the phase of their flagellate mutants (Stocker, Zinder & Lederberg, 1953). Mutation at another locus, *ah1*, closely linked to (or perhaps a part of) *H1* prevents the formation of phase-1 flagella—so that *ah1*⁻ mutants alternate between a normal, flagellate, phase 2 and a phase 1 in which the bacteria are non-flagellate, and therefore non-motile (Iino, 1961). Another kind of mutation, within *H1* or very closely linked to it, produces the 'phase-1-curly' phenotype (Iino, 1962). Such mutants when in phase 2 produce normal flagella and are normally motile; but when in phase 1 they make functionally deficient flagella with an abnormally short wavelength ('curly').

In transductional crosses between *Salmonella* strains differing in phase-1 and phase-2 antigens, the phase of complete transductants shows that the phase of a bacterium is determined at (or close to) its *H2* locus. It seems that *H2* (or a phase-determinant locus, very closely linked to *H2*) exists in either of two 'states': when it is in the active state, the flagellin specified by *H2* is produced, that specified by *H1* is not produced and the cell is in phase 2; when *H2* is in the inactive state the flagellin specified by *H2* is not produced, that specified by *H1* is produced and the cell is in phase 1 (Lederberg & Iino, 1956). The dependence of the phase of a transductant (and so, presumably, of all other cells) on the 'state' of its *H2* locus is inferred from the observation that the phase of a transductant clone is always that of the parent strain (donor or recipient) from which it derived its *H2* allele. For instance, with an *H1*-linked *fla*⁻ recipient all *fla*⁺ transductants, whether or not they inherit the donor *H1* allele and whatever the phase of the donor, are found to be in phase 1 if the recipient culture was in (latent) phase 1, and in phase 2 if it was in (latent) phase 2. By contrast, transduction of an active *H2* locus from a donor in phase 2 to a motile recipient results in *H2* transductants which are in phase 2, regardless of the phase of the recipient—whereas if the donor is in phase 1, i.e. with an inactive *H2*, transduction of *H2* is undetectable, because the *H2* transductants, having acquired an inactive *H2*, are consequently in phase 1, and so do not express their new phase-2 antigen. Iino & Lederberg (1964) discussed the interpretation of phase variation and related phenomena in terms of current concepts of the regulation of genes which specify proteins. Among other possibilities they suggested that *ah1* might be an operator locus for the *H1* gene which it adjoins. They listed several possible explanations for the epistasis of *H2* to *H1*.

The main evidence for the above interpretation of phase-variation comes from analysis of the flagellar antigens of complete transductants, which are haploid for all loci concerned. Study of the flagellar antigens of cells heterozygous for the *H1* or *H2*

regions would permit examination of dominance and *cis/trans* effects, and allow a test of the ability of a chromosomal *H2* locus to regulate the expression of *H1* and *H2* genes not located in the same chromosome. We now describe an analysis of the flagellar antigens of such partial heterozygotes, obtained by the abortive transduction of the *H1* or *H2* regions. The abortive transduction of motility to non-motile recipient strains results in the appearance of trails, i.e. linear groups of microcolonies extending away from the site of inoculation, when the transduction mixture is incubated on semi-solid medium. Each trail marks the path of the successive descendants carrying a transduced fragment of donor chromosome, which is never replicated but which contains a gene conferring the motile phenotype (Stocker *et al.* 1953; Lederberg, 1956; Stocker, 1956). Antisera reacting with the flagella of a motile bacterium immobilize it, and the flagellar antigen(s) of trail-producing cells can be inferred from the ability of test sera, incorporated in the semi-solid medium, to prevent the formation of trails. Lederberg (1956) thus determined the flagellar antigens of abortive *fla*⁺ transductants in a monophasic strain. We used diphasic donor and recipient strains. The recipient strains used were non-motile, either because they were *fla*⁻ mutants, or because they were phase-1 cultures of strains which are non-motile when in this phase (*ahI*⁻ or 'phase-1-curly'). Some such experiments are described elsewhere (Pearce & Stocker, 1965) as evidence that the chromosome fragments transduced by phage P 22 are not, as formerly supposed, invariant in composition.

METHODS

Bacterial strains. The strains used were all *Salmonella typhimurium* and, except where the contrary is stated, were derivatives of strain LT2, most of them with various nutritional and drug-resistance mutations, not here listed because not relevant. In some, one or both of the wild-type flagellar antigen determinants of *S. typhimurium*, *H1-i* and *H2-1,2*, had been altered by mutation, or replaced by transduction.

The non-flagellate mutants used were *fla-56* and *fla-57*, of complementation group A, and *fla-58*, of complementation group D (Joys & Stocker, 1965). All three strains when treated with phage P 22 grown on LT2 *fla*⁺ produce many long trails, and their *fla* sites are closely linked to *H1* (rate of co-transduction of *H1* with *fla*⁺ > 0.02 Joys & Stocker, 1965; Pearce & Stocker, 1965). These *fla* mutants were obtained in lines in which the wild-type phase-1 antigen, *i*, had been modified by mutation at *H1* (Joys & Stocker, 1966)—but the relatively minor serological differences between the modified and wild-type antigen are of no significance for the present purpose.

The *ahI*⁻ strains used were two of the three non-identical, non-complementing *ahI*⁻ strains described by Iino (1961). Strain SL870 is an auxotrophic derivative of Iino's strain SW 1061, which is a spontaneous *ahI*⁻ mutant of LT2; we shall indicate its mutant allele as *ahI-1*. SW 547 is a naturally occurring *ahI*⁻ strain, not derived from strain LT2 (Iino, 1961); we shall indicate its mutant allele as *ahI-3*.

The 'phase-1-curly' mutant used was SW 577, a *Salmonella typhimurium* strain (not derived from LT2) shown by Iino (1962) to be normally motile and with normal flagella when in phase 2, but almost non-motile and with short-wavelength flagella when in phase 1. As its abnormal character probably results from mutation in the structural gene for phase-1 flagellin, we shall indicate its genotype as *H1-i(curly)*.

The strains in which the original antigens of *Salmonella typhimurium* had been

replaced by other antigens, derived from strains of other species, had been obtained by transduction using phage P 22, but were non-lysogenic for this phage. The source of the introduced *H1* and *H2* alleles, *H1-b* and *H2-e,n,x*, was *S. abony* strain sw803 (Spicer & Datta, 1959). *H1-a* came from *S. abortus-equi* strain SL23. This strain, like other strains of this species, is stable in phase 2. Furthermore, it carries an (un-expressed) phase-1-curly allele (Stocker, unpublished; Iino, 1962). However, the transductional clone we used though it had the phase-1 antigen *a* which is latent in the donor strain, SL23, did not have its (latent) phase-1-curly character—presumably because the *H1-a(curly)* allele of the donor had recombined with the *H1-i* allele of the LT2 recipient to produce an *H1-a* (non-curly) gene. The uncommon *H1* allele *H1-1,2* came from a monophasic-phase-1 strain of *S. paratyphi* B, strain CDC-15. This allele determines the production of phase-1 flagella which in antigenic character (Lederberg & Edwards, 1953) and amino-acid composition (McDonough, 1965) are indistinguishable from phase-2 flagella of antigenic type 1,2, determined by the common *H2* allele *H2-1,2*.

Transduction. Phage P 22 was used for transduction. The phage was propagated by the soft-agar-layer method on the strain to be used as donor (in some experiments re-isolated from a single colony in phase 1 or in phase 2). Lysates were sterilized by filtration or heat treatment. Recipient strains were used as broth cultures, inoculated from single colonies in the desired antigenic phase—or latent phase, in the case of non-flagellate recipient strains. To effect transduction, phage was added at a multiplicity of 5–10, and the mixture held at 37° for 10 min. Samples of serial decimal dilutions (usually three standard loopful amounts, each about 0.005 ml., on each of two plates for each dilution) were then inoculated on the surface of chilled semi-solid medium (Edwards & Bruner, 1942) in 5 cm. diameter plates. This medium is semi-solid at 37° but because of its high gelatin content it is solid at room temperature. The anti-H sera used, obtained from the Serological Standards Laboratory, Central Public Health Laboratory, Colindale, London, N.W. 9, had been so far as necessary cross-absorbed, and had agglutinating titres of 5000–20,000. The final concentration of a serum in the semi-solid medium used to test for inhibition of trail formation was between $\times 5$ and $\times 10$ its agglutinating titre. Testing of the flagellar antigens of swarms (complete *fla*⁺, etc., transductants) confirmed that the sera at the concentrations used were effective and specific. The presence of trails and swarms was determined after overnight incubation at 37°, and the number of trails per arbitrary volume (6 loopful amounts) of undiluted transduction mixture was calculated from the number of trails seen on plates with few or no swarms. As the number of trails counted was necessarily very small, the estimate of trail frequency was of low precision.

In the genotype descriptions of abortive transductants the genes derived from the donor, present in the exogenote, are indicated by a subscript 1, and those of the recipient by a subscript 0.

RESULTS

Regulation of expression of exogenote H1 by the chromosomal phase-determinant

To test whether the phase-determinant, at or closely linked to *H2*, could regulate the expression of an *H1* allele in an exogenote fragment, we constructed abortive transductants, of constitution *fla*₁⁺ *H1*₁/*fla*₀[−] *H1*₀, from an *H1*-linked *fla* recipient.

Strain *H1-iM12 fla-58* (SL831) was chosen as recipient, because it produces long trails and because *fla-58* is closely linked to *H1* (rate of cotransduction of *H1* with *fla-58* about 16%), so that nearly all transduced chromosomal fragments carrying *fla-58*⁺ carry also the *H1* allele of the donor (Pearce & Stocker, 1965). Broth cultures grown from discrete colonies, of predetermined (latent) antigenic phase, were treated with phage P22 grown on phase-1 and phase-2 cultures of *fla*⁺ donor strains having *H1-a* or *H1-b* as their *H1* allele, and with the *H2* allele *H2-e,n,x*. The ability of the transduction mixtures to produce trails in the presence of various sera and combinations of sera was then tested. Table 1 records the results with the donor strain carrying

Table 1. *Salmonella typhimurium*. Effect of antisera on trails evoked from an *H1*-linked *fla*⁻ recipient, in (latent) phase 1 or phase 2, by P22 lysates of phase-1 and phase-2 cultures of a *fla*⁺ donor with a different phase-1 antigen

Donor was LT2 *fla*⁺ *H1-a H2-e,n,x* (= SL861). Recipient was LT2 *fla-58 H1-iM12 H2-I,2* (= SL831). Multiplicity about 10. Swarms were about one-tenth as abundant as trails; their expressed antigens indicated that the antisera, at the concentrations used, were active and specific. Trails from phase-1 recipient about 4 mm. long, those from phase-2 recipient about 6 mm. long (slightly shorter in the presence of anti-*a* serum, presumably because of a slight non-specific activity of this serum).

Line	Phase and relevant antigen of		No. of trails* in semi-solid medium with				
			No serum	Serum for antigen determined by			Donor and recip. <i>H1</i> (anti- <i>a</i> and anti- <i>i</i>)
	Donor	Recipient		Recip. <i>H2</i> (anti- <i>I,2</i>)	Recip. <i>H1</i> (anti- <i>i</i>)	Donor <i>H1</i> (anti- <i>a</i>)	
<i>a</i>	Ph. 1 (<i>a</i>)	Ph. 2 (<i>I,2</i>)	3000	50	3000	4000	4000
<i>b</i>	Ph. 2 (<i>e,n,x</i>)	Ph. 2 (<i>I,2</i>)	3000	30	3000	3000	2000
<i>c</i>	Ph. 1 (<i>a</i>)	Ph. 1 (<i>i-M12</i>)	3000	3000	1	9	0
<i>d</i>	Ph. 2 (<i>e,n,x</i>)	Ph. 1 (<i>i-M12</i>)	8000	9000	0	8	0

* Calculated no./six loopfuls (about 0.003 ml.) of undiluted transduction mixture.

H1-a; the results with the *H1-b* donor were similar. In the absence of any antiserum the number of trails was about the same for all combinations of phase of donor and recipient. The susceptibility of the trails to antisera varied according to the phase of the recipient culture, but was not affected by the phase of the donor. Thus, when the recipient was in (latent) phase 2 (Table 1, lines *a* and *b*), the number of trails was not obviously reduced by the presence of antiserum for the phase-1 antigen of the recipient, or of antiserum for the phase-1 antigen of the donor, or of antisera for both phase-1 antigens—but was reduced about 99% by anti-*I,2* serum, active on the phase-2 antigen of the recipient. This result indicates that nearly all *fla-58*⁺ abortive transductants arising from recipient cells in phase 2 express their chromosomal *H2* allele; but express neither their chromosomal *H1* allele nor the donor *H1* allele which nearly always accompanies *fla-58*⁺ in the exogenote fragment. The few trails not inhibited by antiserum for the recipient phase-2 allele probably arose from a minority of recipient cells in phase 1; their proportion (about 1%) corresponds approximately to the proportion of phase-1 cells predicted by the rate of change from phase 2 to phase 1 in *Salmonella typhimurium* (Stocker, 1949).

When the recipient culture was in (latent) phase 1, the presence of antiserum for the

recipient phase-2 antigen did not decrease the number of trails (Table 1, lines *c* and *d*); thus, as expected, most or all *fla-58*⁺ abortive transductants arising from phase-1 cells did not express their *H2* allele. The presence of antiserum for the donor phase-1 antigen decreased the number of trails by about 99.7 %, while the presence of antiserum for the recipient phase-1 antigen almost entirely prevented the appearance of trails. This indicated that nearly all *fla-58*⁺ abortive transductants arising from phase-1 cells expressed both their chromosomal *H1* allele and the donor *H1* allele which accompanies *fla-58*⁺ in the exogenote fragment. The few (about 0.3 %) trails unaffected by donor phase-1 antiserum probably resulted from a small proportion of transduced chromosome fragments which contained *fla-58*⁺ but not *H1* (Pearce & Stocker, 1965). The single trail not suppressed by antiserum for the recipient phase-1 allele probably arose from a recipient cell in phase 2. The lack of effect of the phase of the donor on the phenotype of *fla*⁺ abortive transductants is expected if phase is determined only at (or close to) *H2*, since no chromosomal fragment carrying *fla-58*⁺ would carry also the unlinked *H2* region of the donor. It therefore appears that in *fla*⁺ abortive transductants carrying two *H1* alleles they are both expressed if the cell is in phase 1, but neither is expressed if it is in phase 2.

Effect of ahI⁻, in cis and in trans, on the expression of H1

When phase-1 (and therefore non-motile) cultures of *ahI*⁻ recipients are treated with lysates of *ahI*⁺ donors, abortive *ahI*⁺ transductants are detectable because they produce trails. We investigated the effect of antisera on such trails, to see whether an exogenote *ahI*⁺ allele would permit expression of an *H1* allele adjacent to *ahI*⁻ in the chromosome. We used as recipients two *Salmonella typhimurium* strains, with the *ahI*⁻ alleles which we designate *ahI-1* and *ahI-3* (Iino, 1961); in both strains the unexpressed phase-1 antigen is *i*. The *ahI*⁺ strains used as donors all had the same *H2* allele as the recipients, *H2-1,2*. One donor strain, LT2, had the same phase-1 antigen determinant, *H1-i*, as the recipients; the other, UP158, carried *H1-a*. The recipient cultures were in phase 1 and therefore non-motile. Anti-*i,2*-serum, active on the phase-2 antigens of all the strains, was included in the semi-solid medium, to prevent spreading of any cells changing into phase 2, by spontaneous change of phase or by transduction of an 'active' *H2* locus. When the donor carried *H1-i*, determining the same phase-1 antigen as the unexpressed phase-1 antigen of the *ahI*⁻ recipients, the presence of anti-*i*-serum decreased the number of trails from thousands to zero (Table 2, lines *a* and *c*). When the donor carried instead *H1-a*, the presence of anti-*i* serum did not detectably decrease the number of trails, but anti-*a* serum prevented the appearance of any trails (Table 1, lines *b* and *d*). In a similar experiment, strain SD7, *H1-b*, was used as donor, and anti-*b* serum in place of anti-*a*; the results were qualitatively similar. It thus appears that *ahI*⁺ abortive transductants express the *H1* allele adjacent (*cis*) to *ahI*⁺ in the exogenote fragment; but that the *H1* allele adjacent to *ahI*⁻ in their chromosome remains unexpressed, despite the presence of *ahI*⁺ in the exogenote, i.e. in *trans*.

A corresponding result was found when *ahI*⁺ was in the chromosome and *ahI*⁻ in the exogenote. The strain used as recipient was non-motile because of the *H1*-linked *fla-66*, and carried *H1-b* (and *ahI*⁺). Cultures in (latent) phase 1 were treated with P22 lysates of the two *ahI*⁻ strains, and, as a control, of an *ahI*⁺ strain, all having the *H1* allele *H1-i*. Serum for the (latent) phase-2 antigen of the recipient strain was

included in all plates, to prevent production of trails or swarms by any *fla*⁺ transductants arising from recipient cells passing into phase 2. Nearly all transduced fragments carrying *fla*-66⁺ carry also *H1* (Pearce & Stocker, 1965) and, presumably, *ahI*, which is very closely linked to *H1*. Whatever the donor strain, the presence of antiserum for the recipient phase-1 antigen prevented all trail formation (Table 2, lines *e*-*g*), i.e. as expected, all the *fla*⁺ abortive transductants expressed the recipient *H1* allele, adjacent to its *ahI*⁺. When the donor was *ahI*⁺, anti-*i* serum (corresponding to the donor *H1* allele) decreased the number of trails by about 99% (Table 2, line *e*); thus nearly all *fla*⁺ abortive transductants expressed the *H1* allele adjacent to *ahI*⁺ in the *fla*⁺ exogenote fragment. By contrast, when the donor was *ahI*-1 or *ahI*-3 (Table 2, lines *f* and *g*), anti-*i* serum did not detectably decrease the number of trails, nor make them shorter. Thus in all or most of the *fla*⁺ abortive transductants the *H1*-*i* allele adjacent to *ahI*⁻ in the exogenote remained unexpressed, despite the presence of *ahI*⁺ in the chromosome. It appears, then, that an *H1* allele, in the chromosome or exogenote, cannot be expressed unless it is adjacent (*cis*) to *ahI*⁺.

Table 2. *Salmonella typhimurium*. Effect of antisera on trails arising (i) from *ahI*₁⁺/*ahI*₀⁻ *H1*₁⁺/*H1*₀⁻ abortive transductants; (ii) from *fla*₁⁺ *ahI*₁⁻ *H1*₁⁻/*fla*₀⁻ *ahI*₀⁺ *H1*₀⁺ abortive transductants

The *ahI*⁻ recipient cultures were phase 1, and therefore non-flagellate. The *fla*⁻ recipient culture was in (latent) phase 1. Trails derived from SL870 (*ahI*-1) were about 3 mm. long, those from SW547 (*ahI*-3) about 5 mm. long, and those from SL696 (*fla*-66) about 7 mm. long.

Strain (genotype and strain no.)		No. of trails* in semi-solid medium with serum for recipient phase-2 antigen and:			Line
		No phase-1 antiserum	Antiserum for recipient phase-1	Antiserum for donor phase-1	
Recipient	Donor				
(i) <i>ahI</i> ₁ ⁺ / <i>ahI</i> ₀ ⁻ trails					
<i>ahI</i> -1 <i>H1</i> -i (SL870)	<i>ahI</i> ⁺ <i>H1</i> -i (LT2)	7000	0	0	<i>a</i>
	<i>ahI</i> ⁺ <i>H1</i> -a (UPI58)	500	500	0	<i>b</i>
<i>ahI</i> -3 <i>H1</i> -i (SW547)	<i>ahI</i> ⁺ <i>H1</i> -i (LT2)	1300	0	0	<i>c</i>
	<i>ahI</i> ⁺ <i>H1</i> -a (UPI58)	140	160	0	<i>d</i>
(ii) <i>fla</i> ₁ ⁺ / <i>fla</i> ₀ ⁻ trails					
<i>fla</i> -66 <i>ahI</i> ⁺ <i>H1</i> -b (SL696)	<i>fla</i> ⁺ <i>ahI</i> ⁺ <i>H1</i> -i (LT2)	4000	0	40	<i>e</i>
	<i>fla</i> ⁺ <i>ahI</i> -1 <i>H1</i> -i (SL870)	3000	0	6000	<i>f</i>
	<i>fla</i> ⁺ <i>ahI</i> -3 <i>H1</i> -i (SW547)	3000	0	3000	<i>g</i>

* Calculated as in Table 1.

Control of *H2* by the phase-determinant in *H2* abortive transductants

As the locus determining the phase of a bacterium is located either at *H2* or very close to it (Lederberg & Iino, 1956) an abortive *H2* transductant would contain two phase-determinants, one at or near *H2* in the chromosome, the other at or near *H2* in the exogenote. If the regulation of expression of *H2* by the phase-determinant is mediated by a cytoplasmic regulatory substance then in an *H2* abortive transductant either both or neither *H2* allele would be expressed. But if the phase-determinant

regulates directly the expression of the *H2* gene which it adjoins (or of which it forms part) the two *H2* alleles in such a cell would be independently regulated, and one might be expressed while the other was unexpressed. Determination of the flagellar antigens of *H2* abortive transductants should therefore show whether the regulation of *H2* is direct or indirect. A recipient non-motile culture which became motile on transduction of *H2* from a suitable donor would be expected to give also trails, in consequence of abortive transduction of *H2*. As none of the known *fla* or *mot* loci are co-transducible with *H2*, it was necessary to use a strain non-motile through some other cause. In one experiment we used as recipient a phase-1 culture of strain SL870, of genotype *ahI*⁻ *H1-i* *H2-I,2* and therefore non-motile when in phase 1. This culture was treated with P22 lysates of phase-1, and of phase-2, cultures of SL861, a motile LT2 line whose H alleles are *H1-a* and *H2-e,n,x*. All the plates of semi-solid medium contained sufficient anti-*I,2* serum to prevent the spread of any recipient bacteria which became motile by spontaneously passing into phase 2. On this medium the recipient bacteria treated with the phase-1 lysate produced many trails (calculated number from a standard inoculum, about 8000; Table 3, line *a*). The additional presence in the semi-

Table 3. *Salmonella typhimurium*. Effect of antisera on trails evoked from phase 1 (and therefore non-motile) *ahI*⁻ and *H1-curly* recipients by P22 lysates of phase-1 and phase-2 cultures of donor with different phase-1 and phase-2 antigens

Donor was LT2 *H1-a H2-e,n,x* (SL861). The trails produced by SL870 were about 3 mm. long; those produced by SW577 about 4 mm. long.

Recipient (genotype and strain no.)	Donor, phase and relevant antigen	No further serum	No. of trails* in presence of serum against H antigen(s) determined by recip. <i>H2</i> and:					Line
			Recip. <i>H1</i> (anti- <i>i</i>)	Recip. <i>H1</i> and donor <i>H1</i>		Donor <i>H2</i> and donor <i>H1</i>		
				(anti- <i>i</i>) + anti- <i>a</i>)	(anti- <i>i</i>) (anti- <i>e,n,x</i>)	(anti- <i>e,n,x</i>) (anti- <i>e,n,x</i>) + anti- <i>a</i>)	(anti- <i>e,n,x</i>) (anti- <i>e,n,x</i>) + anti- <i>a</i>)	
<i>H1-i ahI-I H2-I,2</i> (sl870)	Ph. 1 (<i>a</i>)	8000	8000	12	8000	0	<i>a</i>	
	Ph. 2 (<i>e,n,x</i>)	2000	3000	600	2000	0	<i>b</i>	
<i>H1-i curly H2-I,2</i> (sw577)	Ph. 1 (<i>a</i>)	0	0	2	0	0	<i>c</i>	
	Ph. 2 (<i>e,n,x</i>)	700	800	800	0	0	<i>d</i>	

* Calculated as in Table 1.

solid medium of serum against the phase-2 antigen of the donor, or of antiserum for the unexpressed phase-1 antigen of the recipient, did not sensibly diminish the number of trails. The presence of anti-*a* serum, specific for the phase-1 antigen of the donor, decreased the number of trails from about 8000 to 12. We infer that nearly all the trails evoked by the phase-1 lysate arose from abortive transductants with no flagellar antigen other than that determined by the *H1* allele of the donor—the expected phenotype for *H1₁ ahI⁺/H1₀ ahI⁻* abortive transductants in phase 1. In this experiment since both donor and recipient were in phase 1, all (or nearly all) *H2* abortive transductants would contain two ‘inactive’ *H2* alleles (and an *H1* allele unexpressed because of *ahI*⁻) and would therefore remain non-flagellate and not produce trails. However, a very few trails (12 amongst 8000) were not inhibited by antiserum for the donor phase-1 antigen, and these few trails were inhibited when antiserum for the

donor phase-2 antigen was also present. It appears that though the donor was in phase 1 at the time of lysis, a small proportion (12/8000) of the *H2* abortive transductants expressed the donor phase-2 antigen. This proportion corresponds approximately with the proportion of phase-2 cells calculated to have been present in the phase-1 donor culture at the time it was infected with phage P22.

A different result was obtained when the phase-2 donor lysate was used (Table 3, line *b*). The presence of antiserum against the donor phase-1 antigen decreased the number of trails, but only by about 70 % (from about 2000 to about 600), instead of by more than 99 %. We infer that only about 70 % of the trails evoked by the phase-2 lysate arose from *ahr*⁺ *H1* abortive transductants. When antiserum for the donor phase-2 antigen was present, as well as antiserum for the donor phase-1 antigen, no trails were produced. We infer that the 30 % of trail-forming cells which did not manifest the donor phase-1 antigen manifested instead the donor phase-2 antigen, in consequence of abortive transduction of *H2*. These trails were produced in the presence of antiserum for the recipient phase-2 antigen, added to prevent spread of recipient bacteria becoming motile by spontaneous passage into phase 2. The trail-producing cells concerned therefore showed the donor phase-2 antigen but not the recipient phase-2 antigen. Thus an exogenote *H2* transduced from a donor in phase 2 expressed itself in a recipient cell which was in phase 1, despite the presence of its 'inactive' chromosomal *H2*, which remains unexpressed.

Control of the expression of endogenote H1 by exogenote H2

The phase-determinant at or near *H2* controls the expression of *H1* via the cytoplasm (or more precisely not through the chromosome) presumably by controlling the production of a regulatory substance. Such a regulatory substance might be either a repressor of the activity of *H1*, produced only in cells in phase 2, or a positive regulator (internal inducer) of *H1*, produced only in cells in phase 1. In an abortive *H2* transductant having one phase-determinant in the phase-1 state and the other in the phase-2 state, the chromosomal *H1* gene would be unexpressed if the regulatory substance were a repressor—because the phase-determinant which was in the phase-2 state would determine production of the *H1*-repressor substance. If, on the other hand, the regulatory substance were an 'internal inducer', the chromosomal *H1* gene of such a cell would be induced by the inducer substance determined by the phase-determinant which was in the phase-1 state. The experiments just described (Table 3, lines *a* and *b*) do not distinguish these hypotheses because the *ahr*[−] character of the recipient strain would in any event prevent expression of its *H1* allele. We therefore wished to make similar experiments with an *ahr*⁺ recipient; but it was necessary that this recipient, though in phase 1 and able to express its phase-1 antigen, should not spread through the semi-solid medium, even in the absence of homologous antiserum. We achieved this by use as recipient of a strain with the 'phase-1-curly' character. Such strains when in phase 2 produce normal flagella and spread normally, but when in phase 1 they make flagella with an abnormally short wavelength, show very little translational motility and spread very slowly or not at all in semisolid medium (Iino, 1962). Both genetical evidence (Iino, 1962; Joys & Stocker, 1963) and chemical evidence (Enomoto & Iino, 1966; Asakura, Eguchi & Iino, 1966) indicate that the phase-1-curly (or phase-2-curly) character arises from mutation within the structural gene for the corresponding phase-1 (or phase-2) flagellin, causing a change in the amino acid sequence of this

protein. Theoretical considerations, and observations on the trail-forming ability of $fla^{+1} H1-curly_1/fla^{-0} H1-curly^{+0}$ abortive transductants, described below, indicate that abortive transductants synthesizing both an abnormal ('curly') flagellin and a normal flagellin make flagella containing both proteins, and that such bacteria show translational motility and produce trails. These, however, are abnormally short, presumably because flagella made from the mixture of normal and abnormal protein are less efficient locomotor organs than those containing only normal flagellin. Hence, the abortive transduction of an 'active' *H2* allele to a phase-1 (and therefore non-motile) culture of a phase-1-curly recipient would be expected to confer motility, and so to cause the appearance of trails inhibitable by antiserum for the donor phase-2 antigen; but if in such abortive transductants the chromosomal *H1-curly* allele was also expressed the trails would be abnormally short, and inhibitable also by antiserum for the recipient phase-1 antigen. We used these two criteria to test for expression of the chromosomal *H1-curly* gene in such abortive *H2* transductants, i.e. when the phase-determinant of the recipient was in the phase-1 state and that of the donor in the phase-2 state. The phase-1-curly recipient was SW 577. A phase-1 culture was treated with phase-1 and phase-2 lysates of an LT2 line with the *H* alleles *H1-a* and *H2-e,n,x*. The semi-solid medium contained anti-1,2 serum, specific for the phase-2 antigen of the recipient, to prevent the spreading growth of any recipient bacteria which might change into phase 2. When the phase-1 lysate was applied, only 2 trails were detected, both of them on medium containing antisera for the donor and for the recipient phase-1 antigens (Table 3, line *c*). The phase-2 lysate evoked numerous trails (Table 3, line *d*), of average length about 4 mm., i.e. not abnormally short as they would have been if the recipient *H1* allele had continued to direct the synthesis of an abnormal flagellin. Neither the number nor the length of the trails was decreased by the presence of antiserum for the phase-1 antigen of the recipient—nor by antiserum for the donor phase-1 antigen. Anti-*e,n,x* serum, corresponding to the donor phase-2 antigen, decreased the number of trails to zero. We infer that all the trails evoked by the phase-2 lysate arose from *H2* abortive transductants, all of them expressing the donor *H2* allele and none of them expressing either the *H1-curly* or the *H2* allele of the recipient. The exogenote *H2* derived from a donor in phase 2 thus expressed itself after introduction into a phase-1 recipient, and prevented the further expression of the previously expressed *H1* allele (in this case, the *H1-curly* allele) of the phase-1 recipient; but the chromosomal *H2* allele remained unexpressed. This shows that the phase-determinant regulates the expression of *H1* by determining the production of a cytoplasmic repressor of *H1*, not by controlling the production of a positive regulator (inducer) of *H1*, and confirms the inference that the phase-determinant regulates directly the expression of the *H2* gene which it adjoins or forms part of and does not regulate that of another *H2* allele present in the same cell.

In the experiments just described (Table 3, lines *c* and *d*), no trails expressing a phase-1 antigen or antigens, i.e. no abortive *H1* transductants, were detected, even though in the similar experiment with the *ah1*⁻ recipient (Table 3, lines *a* and *b*) most of the trails were of this sort. The absence of *H1* abortive transductants is probably only apparent and results from the *H1-curly* character of the recipient. Phase-1 cultures of this strain, SW 577, are not entirely non-motile, and they spread very slowly in semi-solid medium. This slow spread does not interfere with the detection of trails of normal length (about 5 mm.). But in experiments with recipients non-motile

because of *fla*⁻ mutation (*fla*-58 or *fla*-66) closely linked to *H1*, the *fla*⁺ abortive transductants evoked by an *H1-curly* lysate made trails which were very much shorter than those evoked by an *H1-curly*⁺ lysate, presumably because they are of constitution *H1-curly*₁ *fla*⁺₁/*H1-curly*⁺₀ *fla*⁻₀ and therefore make flagella containing both abnormal and normal flagellin, and are consequently less motile than bacteria whose flagella contain only normal flagellin. In the experiment in which the recipient was the phase-1-curly strain (Table 3, lines *c* and *d*) *H1* abortive transductants would likewise presumably have produced only short trails, and these might well have been obscured by the narrow halo of spread of the recipient strain.

Consideration of phase-2 flagellin as a possible repressor of H1

Bacteria which are in phase 2 and are flagellate produce both phase-2 flagellin and, we have inferred, a repressor of the expression of *H1*. A simple explanation would be that some of the phase-2 flagellin synthesized reaches *H1* via the cytoplasm and acts as the repressor of this gene. We tested this hypothesis by use of an exceptional strain of *Salmonella paratyphi* B, SW 546 = CDC 157, which has an *H1* allele determining flagellin of antigenic character 1,2 (Lederberg & Edwards, 1953), though antigen 1,2 in other strains of *S. paratyphi* B, *S. typhimurium* and many other *Salmonella* species is a phase-2 antigen, determined at the *H2* locus. The 1,2 flagellin determined by the exceptional *H1* allele cannot be distinguished from ordinary 1,2 flagellin, determined by *H2*-1,2, either serologically (Lederberg & Edwards, 1953) or in amino-acid composition (McDonough, 1965), and it seems likely that the flagellins determined by *H1*-1,2 and by *H2*-1,2 are identical. If the repressor of *H1* in ordinary diphasic strains of *S. paratyphi* B, etc., is their phase-2 flagellin 1,2, then the supposedly identical 1,2 flagellin determined by *H1*-1,2 should likewise repress the expression of a normal *H1* allele. To test this prediction, we made phase-1 abortive transductants of constitution *H1*-1,2₁ *fla*⁺₁/*H1*-b₀ *fla*⁻₀ and determined their flagellar antigen phenotype. The donor strain was an LT2 line given the *H1*-1,2 allele by transduction, and with the *H2* allele *H2*-e,n,x. The recipient was a phase-1 culture of an LT2 line of genotype *H1*-b *fla*-66 *H2*-e,n,x. and nearly all phase-1 *fla*⁺₁/*fla*-66₀ abortive transductants express both the donor and the recipient phase-1 antigens. In the experiment with the *H1*-1,2 donor, more than 99 % of the trails were inhibited by anti-1,2 serum, and more than 99 % of them were also inhibited by anti-b serum. That is, the *fla*⁺ abortive transductants expressed both their phase-1 antigens and the 1,2 flagellin determined by the exogenote *H1*-1,2 did not repress the expression of the normal *H1*-b allele in the chromosome. It is therefore unlikely that in ordinary diphasic strains the 1,2 (or other phase-2) flagellin is the repressor substance for *H1*.

DISCUSSION

Consider first the expression of *H1* as influenced by *ah1* and by *H2*. In *ah1*⁺ abortive transductants obtained from a phase-1 *ah1*⁻ culture, the *H1* allele adjacent to *ah1*⁻ in the recipient was unexpressed, though the *H1* allele adjacent to *ah1*⁺ in the exogenote was expressed (Table 2, lines *a-d*). Similarly in phase-1 *fla*⁺ abortive transductants the *H1* allele accompanying *fla*⁺ in the exogenote was unexpressed if it was adjacent to *ah1*⁻, but expressed if adjacent to *ah1*⁺ (Table 2, lines *e-g*). The non-expression of an *H1* allele adjacent (*cis*) to *ah1*⁻ despite the presence of an *ah1*⁺

allele in the same cell shows that *ahI* does not affect the expression of *H1* by means of a cytoplasmic regulatory substance, nor by coding for a product, such as a specialized ribosome, needed for expression of *H1* though not of *H2*. Nonsense or frame-shift mutations within the structural gene for phase-1 flagellin would account for the *ahI*⁻ phenotype. However, *ahI* seems to be distinct from *H1* in that *ahI*⁻ mutants retain the genetic information for making the flagellar antigen determined by their *H1* allele, as is revealed by the phase-1 flagellar antigen of their *ahI*⁺ revertants and of recombinants in which there has been crossing-over between the determinant of phase-1 antigenic specificity and *ahI* (Pearce & Stocker, unpublished observations). The site of *ahI* mutation may be an *H1* 'operator' locus, comprising DNA whose function is to recognize and react with the *H1* repressor substance. The normal phase variation of the exceptional strains which have antigen 1,2 as a phase-1, instead of as a phase-2, antigen suggests that they have a normal *H1* operator, which does not form part of the flagellin structural gene. However, it is possible that the repressor recognition site, assumed to be altered in *ahI*⁻ mutants, is within the flagellin structural gene, but in a part of it which codes for an antigenically null region of the polypeptide chain, perhaps at or near its N terminus.

The experiments on the flagellar antigens of abortive transductants from an *H1*-linked *fla*⁻ recipient (Table 1) tested the ability of the phase-determinant of the recipient to regulate a non-chromosomal *H1* allele. Antiserum for the donor phase-1 antigen inhibited trails evoked by lysates of a donor in phase 1 or phase 2 when the recipient was in latent phase 1, but not when it was in latent phase 2. Thus expression of the exogenote *H1* was controlled by the phase of the recipient, i.e. by the 'state' of its phase-determinant at *H2*, just as was that of the chromosomal *H1* allele. The non-expression of the chromosomal *H1* allele in *H2* abortive transductants evoked from a phase-1-curly recipient strain in phase 1 by a lysate of a donor in phase 2 (Table 3, line *d*) shows that the expression of a previously active chromosomal *H1* allele is prevented by an exogenote active *H2* allele. These observations show that the control of expression of *H1* by the state of *H2* (otherwise stated, the epistasis of *H2* to *H1*) does not depend on their locations on the chromosome, causing, for instance, more *H2* than *H1* messenger RNA to be made (Iino & Lederberg, 1964). They can be explained by several sorts of cytoplasmic interaction. For instance, *H2* messenger RNA, made only by cells with an active *H2* allele, might compete successfully with *H1* messenger RNA for attachment to a specialized ribosome used in synthesis of flagellin (Iino & Lederberg, 1964). However, analogy with other systems makes it plausible to suppose that the expression of *H1* is determined by an *H1* regulatory substance, whose production or non-production is determined at, or close to, *H2*. This regulatory substance might be either a positive regulator ('internal inducer') such as is involved in the regulation of the arabinose genes in *E. coli* (Englesberg *et al.* 1965)—or a repressor, such as that which regulates the lactose genes in the same organism. Either kind of regulatory mechanism would account for the control of exogenote *H1* by endogenote *H2*. However, the non-expression of the previously active *H1* allele in the *H2* abortive transductants evoked from a phase-1 culture of a phase-1-curly recipient indicates that the regulation of *H1* is effected by a repressor substance, produced by active *H2* regions—for if it was effected by a positive regulatory substance the inactive (since unexpressed) *H2* in the chromosome of the abortive transductants would presumably have continued to determine production of the

'inducer' of *H1*, which would therefore have been expressed. It therefore appears that *H2* does not control *H1* by controlling production of a positive regulatory substance but, probably, by controlling production of a repressor of expression of *H1*.

The expression of the donor phase-1 antigen when an *H1* allele from a phase-2 donor was present in the exogenote in a *fla*⁻ recipient in phase 1, and the non-expression of an *H1* exogenote allele derived from a phase-1 donor in a phase-2 cell of the same recipient strain indicates that regulation of expression of an *H1* allele can be effected by the phase-determinant in the recipient cell even though the *H1* allele concerned is never replicated. This parallels other examples, where environmentally determined repression or de-repression of a gene or operon is not dependent on its replication.

Consider now expression of *H2*. The *H2* abortive transductants evoked from phase-1 cultures of *ah1*⁻ and phase-1-curly recipients by treatment with phase-2 lysates expressed the donor *H2* allele present in the exogenote, but the recipient *H2* allele in the chromosome remained unexpressed. Thus, the expression of an *H2* gene depends on its inherent state (or on the state of its adjacent (*cis*) phase-determinant locus) and is unaffected by the state of another *H2* region in the same cell. This is incompatible with the suggestion (Klein, 1964) that the non-expression of *H2* in phase-1 cells results from the presence in them of a cytoplasmic repressor of *H2*. It suggests, on the contrary, that there is an *H2* operon with two metastable states, the active or phase-2 state corresponding to wild-type and the inactive or phase-1 state to that of an operator-negative mutant.

We have argued above that *H2*—or a phase-determinant locus closely linked to it—regulates expression of *H1* by determining production or non-production of an *H1*-repressor substance. The simplest hypothesis, that the *H1*-repressor substance is *H2* messenger RNA or *H2* flagellin, is not supported by our observation that an ordinary *H1* allele in the chromosome and an exceptional *H1* allele, determining what is usually a phase-2 flagellin, are both expressed in *fla*⁺ abortive transductants. If the messenger RNA and 1,2 flagellin products of the uncommon *H1-I,2* allele are identical with those of the common *H2-I,2* allele then the chromosomal *H1* allele, *H1-b*, would have been repressed if *H2* messenger RNA or *H2* flagellin is the repressor of *H1*. However, the evidence for the identity of flagellin 1,2 determined by *H1-I,2* with that determined by *H2-I,2* does not exclude the possibility of a minor difference in amino-acid sequence, at one end, for instance, which might be antigenically null but affect repressor activity. However, we consider it more likely that there is a special repressor substance, probably a protein, coded for at an *H1*-repressor locus closely linked to *H2* and forming part of the same operon as it. It is then necessary to postulate that the activity or inactivity of this *H2* operon depends on the state of a metastable operator region, which alternates between an 'on' or 'active' configuration, corresponding to wild-type, and an 'off' or 'inactive' configuration, corresponding to that of an operator-negative mutant. It seems unlikely that the postulated metastable region is comprised within the structural gene for phase-2 flagellin, because the exceptional *H1* allele *H1-I,2*, which presumably arose by translocation of an ordinary *H2-I,2* gene, is not metastable. A gene which alternates in a random or mutation-like manner between two alternative states has been reported in *Neurospora crassa* (Barnett & de Serres, 1963). As in the case of the *H2* operator it is not known whether the change from one state to the other depends on a base-substitution or on some local rearrangement, for

instance, an inversion. So far as we know, an operon or unit of expression comprising both a structural gene and a repressor for an unlinked gene has not previously been reported. Such an element might well be involved in the complex regulatory processes of differentiation in higher organisms. The phenomenon of perithecial-aperithecial variation in *Aspergillus nidulans* may depend on the intermittent expression of a gene which determines some cytoplasmic factor affecting perithecial development (Croft, 1967).

The above interpretation of phase-variation requires some modification of the proposals of Iino & Lederberg (1964) on the evolution of this phenomenon. The existence of numerous *Salmonella* species without an *H2* locus, and the homology of the single flagellar antigen locus *H* of the monophasic species *Escherichia coli* with *H1* of *Salmonella* (Mäkelä, 1964) suggest that *H2* arose by a duplication and translocation of a primitive *H1* gene. The response of the *H1* of monophasic *Salmonella* and of *H* of *E. coli* to the *H1*-repressor substance determined at or close to *H2* (Mäkelä, 1964) implies that the primitive *H1* locus, and so presumably also its duplication which became *H2*, had adjacent to it an operator which responded to an *H* regulatory substance. The non-production of flagella (of either phase) in certain environments, e.g. at 44° in *S. typhimurium* or on simple defined medium in *S. typhi*, and the apparent absence of flagellin in the cytoplasm of mutants lacking flagella because of mutation at any of several *fla* loci (Iino & Enomoto, 1966) suggest that there is a regulatory system governing expression of both *H1* and *H2*—probably involving a repressor substance active on both loci. No gene for production of such a repressor of both *H1* and *H2* has been discovered or mapped—but analogy with the *lac* cluster and the presence near *H1* of many genes concerned with various flagellar characters suggest that in a primitive monophasic species a regulatory gene might have been adjacent to *H1*, and therefore involved in the duplication and translocation which produced *H2*. The repressor-recognition site (operator?) of *H2*, and the postulated *H2*-linked duplication of the *H* regulatory gene *H1* might then have changed in such a way that the product of the *H2*-linked regulatory gene continued to repress *H1* but no longer repressed *H2*—thus accounting for the origin of the *H2*-linked *H1*-repressor locus. A local rearrangement at the *H2* region might then have brought this *H1*-repressor locus, previously merely linked to *H2*, into the same operon as it—the situation which we now postulate. If the ability to manifest alternately two different flagellar antigens were for unknown reasons advantageous, then mutations which altered the operator region of the *H2* operon in such a way that it spontaneously alternated between metastable ‘on’ and ‘off’ configurations would be preserved, and would account for phase-variation.

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