Studies on H-O Variants in Salmonella in Relation to Phase Variation

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SUMMARY

Mutants showing oscillatory variation between H-type carrying phase-1 antigen and O-type were isolated from a diphasic derivative of Salmonella typhimurium. The rates of variation from the H-type to O-type were about $10^{-4}$/bacterial division and those in the reverse direction $10^{-3}$ to $10^{-4}$, which are very similar to the rates of variation from the phase-1 to phase-2 of the parent strain and vice versa. Transduction analyses showed that their mutation sites were in $H_2$ (the phase-2 flagellar antigen gene). In O-type bacteria the expression of both the endogenote $H_1$ (the phase-1 flagellar antigen gene) and the exogenote $H_1$ introduced by abortive transduction were repressed. These results indicate that the bacteria are flagellate (H-type) in phase-1 and non-flagellate (O-type) in phase-2, i.e. they are $H_1$-repressor-positive phase-2 non-flagellate mutants (H-O variants). The occurrence of H-O variants strongly supports the hypothesis that there is a special $H_1$-repressor gene in the $H_2$ operon which is concerned with the regulation of $H_1$ expression in phase-2 bacteria.

INTRODUCTION

Most of the Salmonella species show two types of flagellar (H) antigen – phase 1 and phase 2. A bacterium of the phase corresponding to either of these two types of flagellar antigen segregates a minority of bacteria in the other phase. The rate of interchange between two phases ranges from $10^{-3}$ to $10^{-5}$/bacterial division (Stocker, 1949; Mäkelä, 1964; Iino, 1969). This intraclonal change between the two phases is called phase variation, and a strain showing phase variation is called a diphasic strain. The antigens of phase-1 and phase-2 flagella are determined by two genes, $H_1$ and $H_2$, which are located far apart on the chromosome (Smith & Stocker, 1962; Mäkelä, 1964). $H_1$ and $H_2$ are the structural genes for phase-1 and phase-2 flagellin, the protein constituting the flagellar filaments of phase-1 and phase-2 bacteria, respectively. Thus, phase variation can be described as the alternative expression of $H_1$ or $H_2$ (Lederberg & Edwards, 1953).

Lederberg & Iino (1956) suggested that in the process of phase variation $H_2$ is either active or inactive and the state of $H_2$ determines the phase of the bacterium. When $H_2$ is in the active state, the production of phase-2 antigen by $H_2$ proceeds, that of phase-1 antigen by $H_1$ is repressed and thus the bacterium is in phase 2. When $H_2$ changes to the inactive state the production of phase-2 antigen stops, that of phase-1 antigen starts and the bacterium becomes phase 1.

In addition to $H$ genes several other genes involved in phase variation have been identified.
Table I. Bacteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phase-1</th>
<th>Phase-2</th>
<th>Characteristics and derivation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sw1061</td>
<td>(i)</td>
<td>1,2</td>
<td>O-H variant derived from Salmonella typhimurium T2M</td>
<td>Iino, 1961a</td>
</tr>
<tr>
<td>s1w2</td>
<td>g, t</td>
<td>1,2</td>
<td>Diphasic derivative of sw1061 with H1-g, t transduced from Salmonella abortusequii strain 8925</td>
<td>Yamaguchi et al. 1972</td>
</tr>
<tr>
<td>s814</td>
<td>(i)</td>
<td>1,2</td>
<td>O-H variant derived from Salmonella typhimurium SJ770. Phase-2 flagella are straight and therefore non-motile</td>
<td>Iino &amp; Mitani, 1967</td>
</tr>
<tr>
<td>sw803</td>
<td>b</td>
<td>e, n, x</td>
<td>Diphasic. Wild-type Salmonella abony</td>
<td>Spicer &amp; Datta, 1959</td>
</tr>
</tbody>
</table>

(i) = unexpressed antigen type.

Ahr and ah2, closely linked to H1 and H2 respectively, determine the activity of their adjacent H genes and are termed activity controllers (Iino, 1961a, 1962, 1969). The relationship between an activity controller and its adjoining H gene may be analogous to that between the operator and structural genes in an operon. Mutation from ahr+ to ahr− results in inactivation of H1 and the bacteria become non-flagellate in phase 1. As an ahr− mutation does not affect the state of H2, such a mutant shows O–H variation in place of phase variation, i.e. it is non-flagellate in phase-1 (O-type) and flagellate in phase 2 (H-type). On the other hand, mutation from ah2+ to ah2− results in both inactivation of H2 and loss of repression of H1, with the result that the mutant is stable in phase 1. Another gene, vh2, controls the state of H2. Mutation from vh2f to vh2− results in stabilization of the H2 locus in whatever state it is at the time of the mutation (Iino, 1961b).

Two questions about phase variation remain, namely how does vh2 regulate the oscillatory state of H2, and how is the expression of H1 repressed in the H2-active state? Several working hypotheses concerning the first problem have been proposed (Iino, 1969), but no final conclusion based on experimental data is available. As for the second problem, it was shown that active H2 controls the expression of H1 via a cytoplasmic repressor substance, i.e. H2 acts as the repressor of the H1-operon (Iino & Lederberg, 1964). Pearce & Stocker (1967) modified this hypothesis proposing that there is a special repressor substance coded for at an H1-repressor locus closely linked to H2 and forming a part of the same operon as H2. This was also suggested by Klein (1964). Isolation of H1-repressor-negative mutants or H1-repressor-positive phase-2 non-flagellate mutants could lead to further clarification of this problem. The former mutants should always produce both phase-1 and phase-2 antigens and the latter should show oscillatory variation between the flagellate (H) state in phase-1 and non-flagellate (O) state in phase-2, ‘H-O variation’. We have isolated such H-O variants from a derivative of Salmonella typhimurium.

METHODS

Bacteria and bacteriophages. Salmonella strains, sw1061, s1w2, s814 and sw803, were used (Table 1). Bacteriophage χ was used for the selection of H–O variants. The phage lysed various Salmonella strains only when they are motile (Meynell, 1961) and has often been used for the selection of non-motile Salmonella mutants (Joys & Stocker, 1965; Iino & Enomoto, 1966; Yamaguchi, Iino, Horiguchi & Ohta, 1972). Phage P 22 was used for trans-
H-0 variants in Salmonella

Phage was propagated on host bacteria by the soft agar layer method (Adams, 1959).

**Media.** Nutrient broth comprised 1% (w/v) peptone and 1% (w/v) meat extract at pH 7.2. Nutrient agar was prepared by the addition of 1.5% (w/v) agar to the nutrient broth. Semisolid medium was 0.3% (w/v) agar and 8% (w/v) gelatine in nutrient broth. When necessary, antiflagellar serum was added to semisolid medium at a high enough concentration to prevent the spreading growth of motile bacteria with the homologous antigen. All incubations were at 37°C.

**Isolation of H-O variants.** An H-O variant clone originating from a phase-2 bacterium of a diphasic strain was expected to comprise a majority of non-flagellate phase-2 and a minority of flagellate phase-1 bacteria and therefore to form a compact colony surrounded by a halo-like swarm on semisolid medium. The diphasic strain SJW2 g,t:i,2 was used as parent. A 0.5 ml broth culture inoculated with a single phase-2 colony was incubated for 2 h and then 0.05 ml of a suspension of phage χ (about 10^{11} plaque-forming units/ml) propagated on SJW2 were added. After overnight incubation, during which most of motile bacteria were killed by the phage, a portion of the mixed culture was spread on semisolid medium with a platinum loop to give well-isolated colonies and incubated overnight. More than 90% of the surviving bacteria produced compact colonies (non-motile mutants), about 5% produced swarms (wild-type bacteria), and a further 5% produced compact colonies surrounded by a halo-like swarm. Revertible fla− (non-flagellate in both phases) and mot− (flagellate but non-motile) mutants as well as H-O variants should form the third type of colony. To distinguish H-O variants from colonies formed revertible fla− or mot− mutants, a portion of each of the haloes surrounding the compact colonies was transferred to semisolid medium containing anti-g,t serum. In this medium H-type bacteria of H-O variants carrying phase-1 g,t-antigen should be immobilized by the antiserum and therefore form compact colonies, while revertants of fla− or mot− mutants, which are diphasic, should form swarms of phase-2 bacteria carrying i,2-antigen. About 2000 clones forming haloed colonies were examined and 29 which formed compact colonies were retained.

**Transduction.** Phage P22 was used for transduction. Abortive and complete motility transductants were selected on semisolid medium (Stocker, Zinder & Lederberg, 1953; Iino & Enomoto, 1966). A lysate of donor phage was mixed with an overnight broth culture of a recipient at a multiplicity of input of 10. After 15 min the mixture, or its 10^{-3} dilution, was quantitatively brushed in lines on semisolid medium with a 0.1 ml glass syringe. When necessary, antiflagella serum or sera were added previously to the semisolid medium to prevent the spreading growth of some bacteria. After overnight incubation the number of swarms and trails was scored. A swarm corresponds to a complete transductant clone; a trail marks the path of the successive descendants of an abortive transductant carrying a non-replicated fragment of donor chromosome (Lederberg, 1956; Stocker, 1956). Trail formation was used as the criterion of complementation between motility genes of the donor and the recipient. The flagellar antigens of complete transductants were typed by slide agglutination. Flagellar antigens of abortive transductants were determined by inhibition of trail formation by specific antisera incorporated in the semisolid medium (Lederberg, 1956; Pearce & Stocker, 1967).

**Electron microscopy.** Samples were fixed in 0.5% formalin, negatively stained with phosphotungstic acid by the methods of Brenner & Horne (1959), and then examined with a JEM-T7 electron microscope.
Table 2. *Rates of variation between H- and O-types in H–O variants*

Rates were determined by the method of Stocker (1949) after incubation for 150 to 200 generations in broth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Change observed</th>
<th>Values obtained in four experiments</th>
<th>Average value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJW2</td>
<td>Phase-1 → phase-2</td>
<td>3.44, 3.22, 3.06, 7.39</td>
<td><strong>4.28</strong></td>
</tr>
<tr>
<td></td>
<td>Phase-2 → phase-1</td>
<td>14.4, 13.7, 13.7, 14.1</td>
<td><strong>14.0</strong></td>
</tr>
<tr>
<td>H-O variants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJW6</td>
<td>H → O</td>
<td>1.82, 1.36, 1.57, 1.60</td>
<td><strong>1.59</strong></td>
</tr>
<tr>
<td></td>
<td>O → H</td>
<td>7.16, 5.19, 9.17, 8.01</td>
<td><strong>7.03</strong></td>
</tr>
<tr>
<td>SJW15</td>
<td>H → O</td>
<td>3.30, 4.52, 4.90, 7.49</td>
<td><strong>5.05</strong></td>
</tr>
<tr>
<td></td>
<td>O → H</td>
<td>4.83, 5.52, 4.93, 18.6</td>
<td><strong>8.47</strong></td>
</tr>
<tr>
<td>SJW17</td>
<td>H → O</td>
<td>3.42, 2.88, 3.38, 2.43</td>
<td><strong>3.14</strong></td>
</tr>
<tr>
<td></td>
<td>O → H</td>
<td>7.55, 9.58, 10.9, 12.8</td>
<td><strong>10.3</strong></td>
</tr>
</tbody>
</table>

**RESULTS**

*Isolation and characteristics of the mutants*

Twenty-nine clones which were thought to be H–O variants were obtained from phase-2 bacteria of the diphasic strain SJW2 g,t:1,2. They formed compact colonies surrounded by halo-like swarms on semisolid medium and compact colonies without the halo on medium containing anti-g,t serum. When 2-day broth cultures of colonies from any of these clones were spread on to nutrient agar, two types of colonies appeared after overnight incubation – those agglutinated by anti-g,t serum and others agglutinated neither by anti-g,t nor anti 1,2 serum. When single colony broth cultures of either type were incubated for not less than 2 days and similarly spread on to nutrient agar, the two types of colonies were again obtained. Electron microscopy showed that those from g,t-type colonies had flagella but those which were of the non-agglutinated type did not. These results indicate that bacteria of these clones are either flagellate (H) or non-flagellate (O), and that a bacterium in a given state segregates bacteria in the other state. The flagellar antigen of H-type bacteria was always phase-1 g,t.

After 48 h of incubation on semisolid medium containing anti-g,t serum, 26 of 29 clones gave 5–400 swarms/1 × 10^8 incubated bacteria. These swarms were shown to be revertant clones showing diphasic variation between g,t-type and 1,2-type having lost the ability to produce O-type descendants. Three stable mutants, SJW6, SJW15 and SJW17, not producing revertants were mainly used in the following experiments.

**Rates of alteration between H- and O-types in the mutants**

The rates of alteration from H-type to O-type and vice versa were determined in SJW6 SJW15 and SJW17 by the method of Stocker (1949). The rates from H- to O-type were about 10^4/bacterial division and from O- to H-type 10^3 to 10^4 (Table 2). These rates are very similar to those for the variation from the phase 1 to the phase 2 and vice versa in the parent strain SJW2.
Transduction of motility character from a diphasic strain of *Salmonella abony* to the O-type bacteria of the mutants

To locate the sites of mutation, transduction of the motility character was carried out from a diphasic strain, *Salmonella abony* sw803 b:e,n,x, to the mutants. A P22 lysate of a phase-2 culture of sw803 was mixed with O-type cultures of the mutants. The mixtures were brushed in lines on semisolid medium containing anti-g,t serum which prevented spreading growth of the recipients which became motile by alteration from O- to H-type. After overnight incubation, 246 to 315 motile transductant clones appearing as swarms were subcultured on nutrient agar plates and their antigens typed (Table 3). All were b- or e,n,x-type and none was I,2-type. To determine whether they segregated O-type bacteria like recipients, 2-day broth cultures of the transductant clones were spread on semisolid medium to give well-isolated colonies, and incubated overnight. All the b-type clones segregated compact O-type colonies, while e,n,x-type clones did not. Inocula from the same cultures were also brushed in lines on semisolid medium containing homologous antiserum (anti-b or anti-e,n,x) to determine whether they segregated motile bacteria of other antigenic types. None of the b-type clones produced any swarms, whereas those of e,n,x-type produced swarms which were agglutinated by anti-g,t serum (Table 3). Thus, in b-type transductants, H1-b from the donor had replaced H1-g,t of the recipients but the potentiality of the recipients to produce O-type descendants was not affected. On the other hand, e,n,x-type transductants had received H2-e,n,x from the donor with the results that they lost the potentiality to produce O-type descendants and became diphasic. From these results it is concluded that the mutation sites of the mutants are in the H2 gene or in a gene very closely linked to it.

Transduction between pairs of mutants

Transductions were carried out between the three stable and three revertible mutants (sjw24, sjw26 and sjw30) in all pairwise combinations. O-type cultures of them were used as both donors and recipients. Phase-2 cultures of sjw2, the parent strain, and sj814 carrying phase-2-straight flagella and therefore non-motile in phase-2, were used as control donors. The mutation site of sj814 responsible for the phase-2-straight character has been shown to be in H2. Transduction mixtures were brushed on semisolid medium containing anti-g,t serum to prevent spreading growth of recipient bacteria which became H-type. After overnight incubation, the plates were examined for the occurrence of swarms and trails (Table 4). No trails were observed in any crosses except those in which sjw2 was donor. As failure of
Table 4. Production of swarms and trails in transductions between O-type cultures of the H-O variants

Transduction mixtures were brushed in lines on semisolid medium containing anti-g, t serum specific to the H-type of the recipients. The numbers of swarms and trails from the mixture of 2 × 10^6 phage particles and 2 × 10^8 bacteria were scored. Phase-2 cultures of sw814 carrying phase-2-straight flagella and sw26, the parent of the H-O variants, were used as control donors. The trail production is recorded in parentheses.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>sww6*</th>
<th>sww15*</th>
<th>sww17*</th>
<th>sww24†</th>
<th>sww26†</th>
<th>sww30†</th>
<th>sw814</th>
<th>sww2</th>
</tr>
</thead>
<tbody>
<tr>
<td>siw6</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>123 (0)</td>
<td>2 (0)</td>
<td>4 (0)</td>
<td>2 (0)</td>
<td>226 (+ + +)</td>
</tr>
<tr>
<td>siw15</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>86 (0)</td>
<td>0 (0)</td>
<td>2 (0)</td>
<td>0 (0)</td>
<td>202 (+ + +)</td>
</tr>
<tr>
<td>siw17</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>105 (0)</td>
<td>66 (0)</td>
<td>21 (0)</td>
<td>0 (0)</td>
<td>210 (+ + +)</td>
</tr>
<tr>
<td>siw24</td>
<td>34 (0)</td>
<td>6 (0)</td>
<td>7 (0)</td>
<td>0 (0)</td>
<td>45 (0)</td>
<td>6 (0)</td>
<td>10 (0)</td>
<td>210 (+ + +)</td>
</tr>
<tr>
<td>siw26</td>
<td>21 (0)</td>
<td>1 (0)</td>
<td>1 (0)</td>
<td>23 (0)</td>
<td>2 (0)</td>
<td>29 (0)</td>
<td>2 (0)</td>
<td>244 (+ + +)</td>
</tr>
<tr>
<td>siw30</td>
<td>14 (0)</td>
<td>3 (0)</td>
<td>14 (0)</td>
<td>9 (0)</td>
<td>78 (0)</td>
<td>0 (0)</td>
<td>7 (0)</td>
<td>246 (+ + +)</td>
</tr>
</tbody>
</table>

(o) = no trails. (+ + +) = large number of trails.

* Stable H-O variants. † Revertible H-O variants.

Trail production means that complementation between donor and recipient resulting in motility does not occur, the results indicate that the mutation sites of all the mutants are in a single functional unit, H2.

The failure of swarm production in transduction means that mutation sites of donor and recipient overlap each other. The data in Table 4 suggest that the mutation sites of the three stable mutants, siw6, siw15 and siw17, overlap each other, and at least two of them, siw15 and siw17, are deletion mutants. The deleted region of siw15 covers mutation sites of siw6, siw26 and siw14 and that of siw17 covers those of siw6 and siw14. Mutation sites of point mutants siw24 and siw30 are located outside these deletions. The recombination data (Table 4) is insufficient to determine the relative positions of the mutant sites from the numbers of swarms in different crosses.

Expression of exogenote H1 introduced into O-type bacteria of the mutants

Pearce & Stocker (1967) showed that expression of an exogenote H1-allele in a phase-2 bacterium was repressed by a cytoplasmic repressor produced by the bacterium. To determine whether the H1-allele in an exogenote introduced into O-type bacteria of the mutants obtained here was also repressed, we tried to construct by transduction partial heterozygotes of the constitution H1unexpressed H1 H2-. If such partial heterozygotes produce flagella they will form trails in semisolid medium, and their flagellar antigens can be inferred from the ability of antisera in the semisolid medium to prevent trail formation (Pearce & Stocker, 1967). Transduction between phase-2 culture of sw803 (carrying H1-b and H2-e,n,x) and O-type cultures of the mutants was carried out. A phase-1 culture of sw1061, an O–H variant, was used as a recipient control. Both H1 and H2 in exogenote fragments introduced from a phase-2 culture of a diphasic strain into O-type bacteria of sw1061 are known to be expressed (Pearce & Stocker, 1967). The P22 lysate propagated on a phase-2 culture of sw803 was mixed with overnight broth cultures of the recipients, and the ability of the mixtures to produce trails in four kinds of semisolid medium containing different antisera was examined (Table 5). All the plates of semisolid medium contained anti-1,2 or
Table 5. Effect of antisera on trails obtained from O-type cultures of H-0 variants treated with a P22 lysate of a phase-2 culture of a diphasic donor with different H1- and H2-alleles

A phase-2 culture of Salmonella abony b:e,n,x was used as donor. An O-type culture of sw1061, an O-H variant of S. typhimurium, was used as control recipient. Transduction mixtures were brushed in lines on semisolid medium containing various antisera. After overnight incubation the numbers of trails from the mixture of $2 \times 10^8$ phage particles and $2 \times 10^9$ bacteria were scored.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>No additional serum</th>
<th>Donor phase 1 (anti-b)</th>
<th>Donor phase 2 (anti-e,n,x)</th>
<th>Donor phase 1 and 2 (anti-b and anti-e,n,x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-H variant sw1061</td>
<td>866</td>
<td>593</td>
<td>358</td>
<td>0</td>
</tr>
<tr>
<td>H-0 variants</td>
<td>965</td>
<td>999</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sw6</td>
<td>769</td>
<td>592</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sw15</td>
<td>526</td>
<td>570</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

anti-g. t serum to prevent the spreading growth of recipient bacteria which became H-type by variation. On these plates all the transduction mixtures produced trails. When sw1061 was the recipient, the inclusion of antiserum against the donor phase-1 antigen b decreased the number of trails by about 60% and that of antiserum against the donor phase-2 antigen e,n,x by about 30%. This means that 30 to 40% of the abortive transductants possessed the donor phase-1 antigen b and the remaining 60 to 70% the donor phase-2 antigen e,n,x, indicating that both H1 and H2 in the exogenote fragments were expressed. When the O-type bacteria of the H-0 mutants were used as recipients, the number of trails was not significantly reduced by the presence of anti-b serum, but they were eliminated by anti-e,n,x serum. Thus, all the abortive transductants possessed the donor phase-2 antigen e,n,x and none the donor phase-1 antigen b, indicating that the exogenote H2 but not H1 was expressed.

**DISCUSSION**

The mutants obtained possessed the characteristics of H1-repressor-positive phase-2 non-flagellate mutants, or 'H-O variants' as defined in the Introduction, i.e. they show oscillatory variation between H- and O-types. H-type bacteria always showed the phase-1 antigen of the parent, and their mutation sites were in H2. In O-type bacteria the expression of H1, both in the endogenote and in the exogenote, was repressed. The occurrence of such mutants strongly supports the hypothesis of Pearce & Stocker (1967) that the repressor regulating H1 expression is not the product of H2 but a substance coded for at an H1-repressor gene closely linked to H2 and forming part of the same operon. The repressor gene distinguished from H2 is designated rhr. However, it is still possible that the product of the mutated H2 (messenger RNA or flagellin) which is defective in producing phase-2 flagella still retains the ability to repress H1 expression.

Ah2 has been assumed to be the operator of H2-operon. However, because of the occurrence of only one known cistron (H2) in the operon, its distinction from H2 has been based solely on recombination between ah2 and the antigen determinant (Iino, 1962). The present experiments give support to the assumption, indicating that ah2 and H2 are different in function from rhr, i.e. an H2- mutation does not affect rhr activity, whereas an ah2- muta-
tion results in the inactivation of both $H_2$ and $rhi$. The occurrence of $H-O$ variation in the $H_2^-$ mutants in contrast to the $ah2^-$ mutants, which are stable in phase-1, further supports the inference that the locus responsible for the phase shift is not $H_2$ but $ah2$.

An abnormal antigenic recombinant which alternatively expresses phase-I antigens of both donor and recipient at the same frequency as that of normal phase variation has been obtained from crosses between *Salmonella typhimurium* and *S. abony* (Iino, 1961 c). From the information obtained here, the recombinant is judged to have originated by the replacement of the recipient $H_2$ by the donor $H1$ without $ah1$ and also without affecting the regulator genes $vh2$ and $rhi$ in the recipient.

We are grateful to Professor T. Taira of Waseda University for his encouragement and helpful discussion.

**REFERENCES**


