Isolation and Genetic Analysis of Operator-constitutive Mutants of the HI Operon in Salmonella typhimurium

By H. FUJITA,† S. YAMAGUCHI,† T. Taira,† T. HIRANO‡ and T. IINO§

1Department of Biology, School of Education, Waseda University, Nishiwaseda, Shinjuku-ku, Tokyo 160, Japan
2Central Research Laboratory, School of Medicine, Jikei University, Nishishinbashhi, Minato-ku, Tokyo 105, Japan
3Laboratory of Genetics, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

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In phase-2 cells of diphasic Salmonella strains, expression of HI is repressed by the HI repressor, coded for by the rhl gene. A procedure for the isolation of operator-constitutive (HI-0*) mutants of the HI operon is described. Using three-factor crosses between an HI-0* HI strain and HI-O+ HI strains, where motility recovery via HI-phase (or phase 1) flagellation was used as the selected marker and the HI-O character was the unselected marker, the relative position of the HI-0* site to the HI gene was determined. A diphasic HI-O* strain produced, in phase 2, copolymer filaments composed of HI and H2 flagellin.

INTRODUCTION

Most Salmonella species carry two structural genes, HI and H2, for flagellin, the protein component of flagellar filaments. The genes are expressed alternately by the mechanism known as phase variation; cells expressing HI or H2 are said to be in phase 1 or in phase 2, respectively (Iino, 1977). In phase variation, two coupled systems are involved (Fig. 1). Expression of H2 is regulated by a site-specific recombination which inverts a segment of DNA (PD or phase determinant) containing the promoter for the H2 operon (Zieg et al., 1977; Zieg & Simon, 1980). The site-specific recombination requires the presence of the vh2 gene (controller for the variation of the state of H2) which is equivalent to hin (Kutsukake & Iino, 1980; Silverman & Simon, 1980). Expression of HI is regulated by a repressor, the product of rhl (repressor of H1), a gene that lies within the H2 operon (Fujita et al., 1973; Silverman et al., 1979). Thus, the expression of HI is determined by the state (active or inactive) of the H2-rhl operon, HI being expressed only when the H2-rhl operon is in the inactive state.

Among the factors involved in phase variation, the operator of the HI operon, to which the rhl product presumably binds, has not yet been identified. In this study, we describe the isolation of operator-constitutive mutants of the HI operon and the approximate location of the operator.

METHODS

Organisms. Salmonella typhimurium strains used are listed in Table 1. Their genotypes are indicated by the following nomenclature. Allelic genes of HI and H2 expressing different flagellar serotypes are indicated as, for example, HI-gt which means that the HI allele expresses flagella of the serotype 'gt'. The wild-type alleles of HI

† Present address: Tokyo Metropolitan Research Laboratory of Public Health, Hyakunin-cho, Shinjuku-ku, Tokyo 160, Japan

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Fig. 1. Mechanism of phase variation. PD, phase determinant (invertible region); P, promoter (the arrow on or under P represents the direction of transcription); O, operator of the H1 operon, which is described as H1-O in this paper.

### Table 1. *Salmonella typhimurium* strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJ814</td>
<td>H1-1[H2-1.2straight rh1+] on-off vh2+</td>
<td>O-H variant producing straight flagella in phase 2 (Iino, 1967)</td>
</tr>
<tr>
<td>SJW796</td>
<td>(H1-gt+)[H2-enx+ rh1+] on-off vh2</td>
<td>Phase-2 stable derivative of TM2; H1-gt+ was transduced from SJ925 (Yamaguchi et al., 1972) and [H2-enx+ rh1+] on-off vh2 from SL23 (Iino, 1962)</td>
</tr>
<tr>
<td>SJW797</td>
<td>H1-gt+ [H2-enx+ rh1+] on-off vh2</td>
<td>Phase-1 stable derivative of SJW796 (Fujita et al., 1981)</td>
</tr>
<tr>
<td>SJW803</td>
<td>H1-gt (H2-enx+ rh1+) on-off vh2</td>
<td>H1 mutant of SJW797 (laboratory stock)</td>
</tr>
<tr>
<td>SJW846</td>
<td>(H1-gt) [PH2-enx rh1+] on-off vh2</td>
<td>Phase-2 stable H2 (ΔH2-134) mutant (Yamaguchi et al., 1984a)</td>
</tr>
<tr>
<td>SJW1250</td>
<td>(H1-gt+) [ΔH2-enx rh1+] on-off vh2</td>
<td>Phase-2 stable H2 mutant of SJW796 (this study)</td>
</tr>
<tr>
<td>SJW1741</td>
<td>H1-Oc H1-gt+ [ΔH2-enx rh1+] on-off vh2</td>
<td>H1-Oc mutant of SJW1250, expressing H1-gt+ constitutively (this study)</td>
</tr>
<tr>
<td>SJW2110</td>
<td>H1-Oc H1-gt+ [H2-enx+ rh1+] on-off vh2</td>
<td>Phase-1 stable H1-Oc strain, prepared by introducing H1-Oc H1-gt+ from SJW1741 into SJW803 (this study)</td>
</tr>
<tr>
<td>SJW2867</td>
<td>H1-Oc H1-gt+ [H2-1.2straight rh1+] on-off vh2+</td>
<td>Diphasic strain, prepared by introducing H1-Oc H1-gt+ from SJW1741 into SJW814 (this study)</td>
</tr>
</tbody>
</table>

* To indicate the state of H1 and H2 operons, a special notation was adopted (see Methods).

and H2 are indicated with the superscript symbol '+' and non-flagellate mutant alleles without it. The H2-rhl operon in Vh2+ (diphasic) strains, in which the operon changes its state between 'on' (active) and 'off' (inactive), is indicated as [H2-rhl+] on-off. Where the operon is fixed in one state owing to a vh2 (or hin) mutation, this is indicated by the superscript symbol 'on' or 'off'. Where the rhl gene is intact but not expressed owing to a polar mutation in the upstream gene, H2, the operon is indicated as [H2 (rhl+)] on-off rhl. Repressed H1 alleles in strains that are stable in phase 2 are parenthesized, as (HI+) [H2-rhl+] on-off. The operator of the H1 operon is indicated as H1-O; the wild-type allele is indicated as H1-O+ and constitutive mutational alleles as H1-O.*

The flagellotropic bacteriophage χ (Meynell, 1961) was used to select non-flagellate mutants. Bacteriophage P22 (wild-type) or P22 int was used as the mediator for transduction.

**Media.** Nutrient broth, nutrient agar (NA) and semisolid medium (NGA) have been described by Yamaguchi et al. (1972). When necessary, anti-filament serum was added to NGA to prevent the spreading growth of bacteria of a specific serotype (Yamaguchi & Iino, 1970).

**Isolation of non-flagellate mutants.** Spontaneous non-flagellate mutants were isolated by the combined use of phage χ and NGA according to Yamaguchi et al. (1972). H1 and H2 mutants were identified by complementation tests with SJW803 H1 and SJW846 H2, respectively.
Constitutive mutants of HI in Salmonella

Original strain: SJW796

\[ H1-O^+(H1^+)(H2^+ rhlh')^+v2 \]

Select H2 mutants using phage \( \chi \)

Non-flagellate H2 mutants (e.g. SJW1250)

\[ H1-O^+(H1^+)(H2rh^1')^+v2 \]

Select motile segregants using NGA plates

Motile segregants (possible genotypes)

\[ (1) H1-O'HI'[H2rh^1']^+v2...H1-O' \]
\[ (2) H1-O'HI'[H2rh^1']^+v2...rh1 \]
\[ (3) H1-O'HI'[H2rh^1']^+v2...polar \]

Identification of H1-O' mutants by transduction to an Rh1' strain, SJW846 (see text)

Fig. 2. Procedure for the isolation of H1-O' mutants.

Reversion was tested by streaking overnight broth cultures of mutants on NGA plates, incubating for 48 h at 37°C, and inspecting for swarms. For testing reversion of H2 mutants with the genotype \( (H1-gt^+)(H2 rh^1+)^+v2 \), NGA plates supplemented with anti-gt serum were used to prevent spreading growth of occasional gt-type, that is, H1-gt', clones.

Transduction experiments. Complementation and recombination between pairs of non-flagellate mutants were examined by P22-mediated transduction on NGA plates, and production of trails (abortive transductants) or swarms (complete transductants) was used as the criterion for complementation or recombination, respectively (Yamaguchi et al., 1986). To obtain P22-sensitive recombinants, P22 int was used and resulting transductants were examined for P22 sensitivity by routine spot tests.

Mapping of H2 mutations. Mutational sites within the H2 gene were determined by transductional crosses using deletion mutants as recipients. Deletion mutants and a map comprising 31 deletion segments have been reported by Yamaguchi et al. (1984a).

Deletion mapping of the H1-gt gene. Using H1 mutants isolated from a phase-1 stable strain, SJW797, a deletion map of the H1-gt gene was prepared by the procedure used to map the H2 gene (Yamaguchi et al., 1984a).

Serological procedures. Antiserum against flagellar filaments were prepared according to Yamaguchi et al. (1972). Serotypes of bacteria were identified by routine slide-agglutination tests.

Electron microscopy. Bacteria grown in broth were harvested by low-speed centrifugation (400 g, 10 min) and suspended in distilled water containing 1% (v/v) formaldehyde. Specimens were negatively stained with 0.5% (w/v) phosphotungstate solution (pH 7.0) and examined in a JEM 100C electron microscope. Labelling of flagellar filaments by antibodies was done according to Asakura et al. (1968).

RESULTS

Preparation of the parent strain for the isolation of H1-O' mutants

We expected that operator-constitutive mutants of the H1 operon could be obtained as motile segregants from a phase-2 non-flagellate strain of the genotype \( H1-O^+ (H1^+)(H2 rh^1+)^+v2 \), in which the H1 gene is intact, but not expressed because of the H1-repressor substance coded for by the rh1 gene. Thus, we attempted to isolate such non-flagellate mutants from the phase-2 stable strain, SJW796 \( (H1-O^+(H1-gt^+)(H2-exn^+ rh^1+)^+v2) \) (Fig. 2).

Using the flagellotrophic phage \( \chi \) as the selecting agent, 1500 spontaneous non-flagellate mutants were isolated from SJW796. However, the non-flagellate phenotype can be caused not only by a mutation in H2 but by a mutation in any of the fla genes. To identify H2 mutants, the non-flagellate mutants were subjected to a complementation test with a known H2 strain, SJW846. As a result, seven clones (designated SJW1248 to SJW1254) were shown to be H2 mutants. By recombination tests with known H2 deletion mutants (Yamaguchi et al., 1984a), their mutation sites were mapped (Fig. 3). SJW1250, the only strain which was shown to carry a deletion (covering segments 6 and 7), was chosen as the parent for the isolation of H1-O' mutants.
Fig. 3. An abbreviated deletion map of the H2-enx gene indicating the mutation sites of H2 strains isolated from Salmonella strain SJW796. Figures represent SJW strain numbers and H2 mutation numbers (italics in parentheses); circled figures represent segment numbers. Mutants above the line indicating the chromosome are derivatives of SJW796, and those under the line are representative deletion mutants used in the mapping of the former mutants. For the full deletion map of the H2-enx gene, refer to Yamaguchi et al. (1984a).

Isolation of H1-Oc mutants

To detect motile segregants of SJW1250, a broth culture (approx. 10^9 cells ml^-1) was streaked in lines (approx. 10 μl per line) on NGA plates. After overnight incubation, two to five motile segregants were observed as swarms around every line. These (100 independent clones in total) were isolated and examined to determine their flagellar serotype; all were gt-type, i.e. H1-gt+. This set would be expected to include not only H1-Oc mutants but also two kinds of H1-O+ clones, namely, rhl mutants and mutants in which rhl is intact but is not expressed because of a polar mutation in H2 (cf. Fig. 2).

H1-Oc mutants among the motile segregants were identified by transduction to a non-flagellate phase-2 strain, SJW846 (H1-O+ (H1) (H2 rhl^+)[^v2] vh2), in which the rhl gene is active. If a donor carries an H1-Oc mutation, the H1^+ allele which is cotransduced with it should be expressed in the recipient Rhl+ cell; if, on the other hand, the donor carries the wild-type operator H1-O+, the H1^+ allele cotransduced with it should be repressed in the recipient cell by the rhl gene product, i.e. the H1 repressor. Because the H2-rhl operon is well separated from the H1 operon on the S. typhimurium chromosome (Sanderson & Roth, 1984), neither rhl nor polar H2 mutations can be cotransduced with the H1 operon by P22. Further, since the H2 deletion in SJW846 covers segments 6 and 7 of the H2 deletion map (Fig. 3) and, so, overlaps the deletion in SJW1250, no H2^+ recombinants can be produced in these transductions. Thus, the production of motile transductants should indicate that the donor is an H1-Oc mutant.

Transduction experiments were carried out between the 100 motile segregants and SJW846. Nineteen donors produced swarms and trails, and so were regarded as H1-Oc mutants; they were given strain numbers from SJW1739 to SJW1757.

The spreading abilities of these H1-Oc mutants varied. After overnight incubation, strains with the greatest spreading ability formed swarms of almost the same size as those of normal phase-1 clones, while strains with the least spreading ability formed swarms about half that size. SJW1741, an H1-Oc mutant forming the largest swarm, together with SJW2110, a phase-1 stable strain derived from it, was used in the following studies.

Location of an H1-Oc mutation relative to H1

It is known that H1 lies between flaV and nm1 at 40 min on the S. typhimurium chromosome (Yamaguchi et al., 1984b; Sanderson & Roth, 1984) and that the H1 promoter lies between flaV and H1 (Szekely & Simon, 1983; Homma et al., 1985). Therefore, the H1-Oc region would be expected to be near the flaV-proximal end of the H1 gene. To confirm this, an H1-Oc mutation (that of SJW1741) was mapped by the following three-factor crosses. First, a deletion map of the H1-gt gene (Fig. 4), comprising 19 segments, was constructed using H1 mutants isolated from a
Fig. 4. Deletion map of the *H1-gt* gene in *Salmonella* strain SJW797. Italic figures and circled figures represent *H1* mutation numbers and segment numbers, respectively. *H1-2430* at segment 2 is the mutation of an *H1-0* HI strain, SJW2130, which was used in three-factor crosses for the mapping of the *H1-0* mutation (refer to Table 2 and Fig. 5).
HI mutation sites that explains the occurrence of the H1+ recombinants in the three-factor crosses shown in Table 2. With this arrangement the occurrence of all the H1+ recombinants in Table 2 can be explained by only two crossovers. ○ and ● represent HI-O+ and HI-Oc, respectively. × and a bar line represent H1 mutation sites. Circled figures represent segment numbers in the deletion map of the H1 gene. Figures in parentheses represent regions within which crossovers may have occurred (cf. Table 2).

Table 2. Determination of the position of an HI-Oc mutation with respect to the H1 gene by three-factor crosses

Transductional crosses were carried out from SJW2130 H1-OcHI-2430 to two H1-O+ H1 strains. H1+ recombinants that appeared as swarms on NGA plates were isolated and their H1-O character (+ or c) was determined as described in the text. The relative position of H1-Oc to H1 mutations, inferred from these results, is shown in Fig. 5. The figures in parentheses represent crossover regions shown in Fig. 5.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>No. of H1+ recombinants</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJW2130 H1-O+HI-2430</td>
<td>SJW1921 H1-O+HI-2257</td>
<td>108 (1, 2) 0 108</td>
</tr>
<tr>
<td>SJW2130 H1-OcHI-2430</td>
<td>SJW1967 H1-O+HI-2293</td>
<td>11 (4, 5) 97 (3, 5) 108</td>
</tr>
</tbody>
</table>

phase-1 stable H1-O+ strain, SJW797 (H1-O+ H1-gt*[H2-enx rh1+]offvh2). Then, H1 mutants were isolated from a phase-1 stable H1-Oc strain, SJW2110 (H1-OcH1-gt*[H2-enx rh1+]offvh2), and the mutation sites were located on the deletion map. Next, three-factor transductional crosses were done between H1 mutants of SJW2110 and of SJW797 using motility recovery (H1-gt+) as the selected marker with the H1-O character (+ or c) as the non-selected marker. The crosses were done from mutants of SJW2110 to mutants of SJW797, using P22 int as the mediator. As the H2-rhl operon was in the 'off' state in both donor and recipient strains, all motile transductants should have been in phase 1. Motile transductants that appeared as swarms were isolated and examined to determine their H1-O character by transducing their H1-0 HI region to the non-flagellate Rhl+ strain, SJW846, as described above. When swarms and trails resulted, the donor clone was judged to be H1-Oc.

The H1 mutations used to provide the most precise determination of the H1-Oc site are shown in Fig. 5, and the results of crosses in Table 2. All H1+ recombinants obtained as a result of the cross from SJW2130 (H1-OcHI-2430) to SJW1921 (H1-O+ H1-2257) were H1-O+, suggesting that the H1-Oc site lies inside (or on the right) of H1-2430. Among H1+ recombinants obtained as a result of the cross from SJW2130 (H1-OcHI-2430) to SJW1967 (H1-O+ ΔH1-2293), about 90% (97 among 108) were H1-Oc and the remainder were H1-O+, indicating that the H1-Oc site is located between H1-2430 and ΔH1-2293 in deletion segment 2. The results obtained from

Fig. 6. Homopolymer flagellar filaments of parental strains and copolymer filaments of a recombinant. (a) Normal filaments on a phase-1 cell of SJW1741. (b) Straight filaments on a phase-2 cell of SJ814. (c) Copolymer filaments on a phase-2 cell of a recombinant, SJW2867. (d, e) Phase-1 normal and phase-2 copolymer (curly) filaments of SJW2867 treated with anti-gt antibody (d) or anti-1.2 antibody (e). Both kinds of filaments were labelled with anti-gt antibody (d), while only curly filaments were labelled with anti-1.2 antibody (e). Bar, 1.0 μm.
other crosses (data not shown) were consistent with the conclusion that the HI-0\(^c\) site is located in segment 2 of the deletion map of the HI gene. The significance of this conclusion will be discussed later.

**Flagellar filaments of a diphasic strain containing an HI-0\(^c\) mutation**

A diphasic strain containing an HI-0\(^c\) mutation should, when in phase 2, express HI and H2 genes simultaneously. To confirm this, we attempted to transduce the HI-0\(^c\) HI-gt\(^+\) region of SJW1741 to SJ814 (HI-i HI-1.2\(_{\text{straight}}\) rh1\(_{\text{on-off}}\) rh2\(_{\text{+}}\)). SJ814 is a diphasic strain exhibiting phase variation between non-flagellate phase 1 and flagellate phase 2, where the phase-2 flagellin is defective and assembles to produce filaments that are straight rather than helical (R-type straight polymorph; Asakura & Iino, 1972). In vitro experiments have shown that flagellin derived by depolymerizing normal and R-type straight filaments can copolymerize to form filaments, the representative wave form of which is 'curly', characterized by a shorter wavelength than normal (Asakura & Iino, 1972; Hotani, 1976). Therefore, if, as a result of the introduction of an HI-0\(^c\) mutation, both HI and H2 are expressed in phase 2 of SJ814, curly filaments should be produced.

Transduction was carried out from SJW1741 to a phase-1 culture of SJ814 using P22 int as the mediator. Motile transductants that appeared as swarms on NGA plates were isolated and examined for flagellar serotype by the slide agglutination test. All 20 clones that were examined were gt-type, i.e. H1-gt\(^+\). To test whether or not they had received the HI-0\(^c\) mutation together with the HI-gt\(^+\) allele, these markers were examined by transduction to the non-flagellate Rhl\(^+\) strain, SJW846, as described above. All transductants generated swarms and trails, indicating that they carried the HI-0\(^c\) mutation. One of them, designated SJW2867, was used for the observation of flagellar characteristics of cells when in phase 2.

Phase-1 (gt-type) cells of SJW2867 were inoculated in broth and incubated for 8 h to allow phase variation. Then a small volume of a 10\(^6\)-fold dilution of the culture was spread on an NGA plate. After overnight incubation, compact colonies with the appearance characteristic of curly flagellar mutants (Iino, 1962; Fujita et al., 1981) appeared together with normal swarms. One clone of each of the two colonial types was examined for flagellar serotype, motility in broth, and flagellar filament shape. Cells of the clone that swarmed normally, were agglutinated by anti-gt serum, showed normal swimming in broth and had normal-shaped flagellar filaments: indicating that they were phase-1 cells expressing H1-gt\(^+\). In contrast, cells from the compact colony were agglutinated by both anti-gt and anti-1.2 \(_{\text{sera}}.\) In broth culture they showed rotational movement and a tendency to form aggregates, both of which are characteristics of curly mutants (Iino, 1962; Fujita et al., 1981). Electron microscopic observation showed that the flagellar filaments of these cells were curly (Fig. 6c; the flagella of both parents are shown in Fig. 6a and b respectively for comparison). Antibody labelling of the curly filaments showed that they were labelled almost homogeneously by both anti-gt and anti-1.2 antibodies (Fig. 6d, e). These results show that, in phase 2, SJW2867 produces filaments composed of H1-gt\(_{\text{normal}}\) and H2-1.2\(_{\text{straight}}\) flagellins, both types of subunit being rather uniformly distributed along the length of each filament.

**DISCUSSION**

The nucleotide sequence of a 400 bp stretch covering the upstream region and part of the beginning region of HI has been determined (Szekely & Simon, 1983). The position of the operator for the HI operon has not, however, been established, because of the lack of operator mutants. We have now isolated such mutants.

Our success in obtaining HI-0\(^c\) mutants depended on the isolation of appropriate parental strains, that is, phase-2 stable H2 mutants preserving Rh1\(^+\) activity. These parental strains were sought among non-flagellate mutants derived from a phase-2 stable strain of the genotype (HI\(^+\))(H2\(^+\) rh1\(_{\text{on-off}}\) rh2\(_{\text{+}}\). The efficiency of isolation was low; less than 0.5\(\%\) (7 out of 1500) of the non-flagellate mutants were those desired.
In this study, one particular H1-O" mutation was examined in detail and assigned to segment 2 of the H1 deletion map. This result does not necessarily mean that the operator region lies within the H1 coding region, since a mutation in the promoter region that prevents transcription of the H1 gene is phenotypically indistinguishable from a mutation in the H1 gene itself, and the H1 mutants used in the deletion mapping are likely to include promoter mutants. We have cloned a DNA sequence that encompasses H1 and adjacent sequences and have determined the sites of base changes in several H1-O" mutants. They were all located between the 7th and 20th bp upstream from the coding region for H1 flagellin (Y. Inoue, K. Kutsukake, S. Yamaguchi & T. Iino, unpublished results). Therefore, 'H1' mutations located upstream of the H1-O" site, e.g. H1-2430 and H1-2257 may be promoter mutations.

In experiments with in vitro copolymerized filaments, a gradient of antibody labelling along the filament has often been observed. This has been attributed to the different rate of polymerization of the two kinds of flagellin (Asakura & Iino, 1972; Kamiya et al., 1980). In contrast, in these experiments in vivo copolymerized filaments of a diphasic strain containing an H1-O" mutation appeared to be labelled uniformly both with anti-gt and with anti-1.2 sera, suggesting that both kinds of flagellin are supplied more or less evenly to each flagellum-forming apparatus, and transported non-preferentially to the tip of the filament where assembly occurs. Using merodiploid strains of Escherichia coli, Silverman & Simon (1974) also showed that antigenically different flagellins formed relatively uniform copolymers.

Some of the H1-O" mutants formed smaller swarms than those of normal phase-1 clones. We interpret this to indicate that their H1-O regions have retained some degree of susceptibility to the H1 repressor. The relationship between the specific base change at the H1-O region and the residual susceptibility to the H1 repressor is now under investigation.

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