Isolation and Genetic Analysis of Operator-constitutive Mutants of the *H1* Operon in *Salmonella typhimurium*

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In phase-2 cells of diphasic *Salmonella* strains, expression of *H1* is repressed by the *H1* repressor, coded for by the *rh1* gene. A procedure for the isolation of operator-constitutive (*H1-O^c*) mutants of the *H1* operon is described. Using three-factor crosses between an *H1-O^c* *H1* strain and *H1-O⁺* *H1* strains, where motility recovery via *H1*-phase (or phase 1) flagellation was used as the selected marker and the *H1-O* character was the unselected marker, the relative position of the *H1-O^c* site to the *H1* gene was determined. A diphasic *H1-O^c* strain produced, in phase 2, copolymer filaments composed of *H1* and *H2* flagellin.

INTRODUCTION

Most *Salmonella* species carry two structural genes, *H1* and *H2*, for flagellin, the protein component of flagellar filaments. The genes are expressed alternately by the mechanism known as phase variation; cells expressing *H1* 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 *H1* is regulated by a repressor, the product of *rh1* (repressor of *H1*), a gene that lies within the *H2* operon (Fujita *et al.*, 1973; Silverman *et al.*, 1979). Thus, the expression of *H1* is determined by the state (active or inactive) of the *H2-rh1* operon, *H1* being expressed only when the *H2-rh1* operon is in the inactive state.

Among the factors involved in phase variation, the operator of the *H1* operon, to which the *rh1* product presumably binds, has not yet been identified. In this study, we describe the isolation of operator-constitutive mutants of the *H1* 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 *H1* and *H2* expressing different flagellar serotypes are indicated as, for example, *H1-gt* which means that the *H1* allele expresses flagella of the serotype 'gt'. The wild-type alleles of *H1*

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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

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

* 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-rhI* operon in *Vh2*⁺ (diphasic) strains, in which the operon changes its state between 'on' (active) and 'off' (inactive), is indicated as [*H2*⁺ *rhI*⁺]^{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 *rhI* gene is intact but not expressed owing to a polar mutation in the upstream gene, *H2*, the operon is indicated as [*H2* (*rhI*⁺)]^{on}. Repressed *H1* alleles in strains that are stable in phase 2 are parenthesized, as (*H1*⁺) [*H2*⁺ *rhI*⁺]^{on}. 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*^c.

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.

Fig. 2. Procedure for the isolation of *H1-O^c* 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 $(H1-gt^+)[H2rh1^+]^{on}vh2$, 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. Antisera 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^c* 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 $H1-O^+(H1^+)[H2rh1^+]^{on}vh2$, 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 ($H1-O^+(H1-gt^+)[H2-enx^+rh1^+]^{on}vh2$) (Fig. 2).

Using the flagellotropic phage χ 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^c* 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-O^c* 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-O^c* mutants but also two kinds of *H1-O⁺* clones, namely, *rh1* mutants and mutants in which *rh1* is intact but is not expressed because of a polar mutation in *H2* (cf. Fig. 2).

H1-O^c mutants among the motile segregants were identified by transduction to a non-flagellate phase-2 strain, SJW846 (*H1-O⁺* (*H1*) [*H2 rh1⁺*]^{on} *vh2*), in which the *rh1* gene is active. If a donor carries an *H1-O^c* mutation, the *H1⁺* allele which is cotransduced with it should be expressed in the recipient *Rh1⁺* 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 *rh1* gene product, i.e. the *H1* repressor. Because the *H2-rh1* operon is well separated from the *H1* operon on the *S. typhimurium* chromosome (Sanderson & Roth, 1984), neither *rh1* nor polar *H2* mutations can be co-transduced 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-O^c* 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-O^c* mutants; they were given strain numbers from SJW1739 to SJW1757.

The spreading abilities of these *H1-O^c* 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-O^c* 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-O^c* mutation relative to *H1*

It is known that *H1* lies between *flaV* and *nml* 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-O* region would be expected to be near the *flaV*-proximal end of the *H1* gene. To confirm this, an *H1-O^c* 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-O^c* *H1* strain, SJW2130, which was used in three-factor crosses for the mapping of the *H1-O^c* mutation (refer to Table 2 and Fig. 5).

other crosses (data not shown) were consistent with the conclusion that the *H1-O^c* site is located in segment 2 of the deletion map of the *H1* gene. The significance of this conclusion will be discussed later.

Flagellar filaments of a diphasic strain containing an H1-O^c mutation

A diphasic strain containing an *H1-O^c* mutation should, when in phase 2, express *H1* and *H2* genes simultaneously. To confirm this, we attempted to transduce the *H1-O^c H1-gt⁺* region of SJW1741 to SJ814 (*H1-i* [*H2-1.2^{straight} rh1⁺ Jon-off vh2⁺*]). 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 *H1-O^c* mutation, both *H1* 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 *H1-O^c* mutation together with the *H1-gt⁺* allele, these markers were examined by transduction to the non-flagellate *Rh1⁺* strain, SJW846, as described above. All transductants generated swarms and trails, indicating that they carried the *H1-O^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 10⁶-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 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. 6*c*; the flagella of both parents are shown in Fig. 6*a* 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. 6*d*, *e*). These results show that, in phase 2, SJW2867 produces filaments composed of *H1-gt^{normal}* and *H2-1.2^{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 *H1* has been determined (Szekely & Simon, 1983). The position of the operator for the *H1* operon has not, however, been established, because of the lack of operator mutants. We have now isolated such mutants.

Our success in obtaining *H1-O^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 (*H1⁺*) [*H2⁺ rh1⁺*]^{on} *vh2*. 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^c* 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^c* 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^c* 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^c* mutation appeared to be labelled uniformly both with anti-gt and with anti-l.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^c* 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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