

Analysis of Promoter Mutations in the Histidine Transport Operon of *Salmonella typhimurium*: Use of Hybrid M13 Bacteriophages for Cloning, Transformation, and Sequencing

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Mutations that cause an increased level of expression of the histidine transport operon were isolated and characterized genetically. Five independently isolated promoter-up mutations were transferred to an M13 hybrid phage that carries the histidine transport operon, and their nucleotide sequences were determined. For all five mutations, the change was the same as the one previously determined for promoter-up mutation *dhuA1*: a C-to-T change in the Pribnow box rendered this region more homologous to the consensus sequence. Methods for enabling *Salmonella typhimurium* to support growth of M13 phage effectively and for easy transfer of chromosomal mutations onto the hybrid phage are presented.

The high-affinity transport system for histidine in *Salmonella typhimurium* is composed of four proteins coded for by four genes that form an operon located at 47 min on the recalibrated map of *S. typhimurium* (1) (Fig. 1). A DNA fragment containing this operon has been cloned (2), and its entire nucleotide sequence has been determined (11). The regulatory region for this operon, *dhuA*, has been defined genetically by mutations that either raise or lower the level of expression of the operon. Deletions that remove this site do not express of the operon unless the deletion places the residual genes under control of a neighboring promoter, such as *argTr* (9, 27). Relatively little is known about the regulation of expression of amino acid transport systems. Attenuation of transcription is an important mechanism for regulation of several amino acid biosynthesis operons (13) and has been implicated as a possible regulation mechanism for a branched-chain amino acid transport system (17). However, analysis of the *dhuA* sequence strongly suggests that attenuation of transcription is not the mechanism by which the histidine transport operon is regulated since this sequence does not present any of the features typical of the regulatory regions of the amino acid biosynthetic operons (10). The histidine transport operon is controlled independently from the histidine biosynthesis operon (Ferro-Luzzi Ames, unpublished data). Its expression is under nitrogen control, being positively regulated together with several other cell proteins involved in the utilization and transport of nitrogen-containing compounds, by nitrogen availability (15, 27). Several interesting features of the nucleotide sequence were singled out as possibly being involved in regulation of the operon (10). Recently, we constructed a variety of Mu d1(Ap^r *lac*) operon fusions, which allowed us to analyze the response of this promoter to several environmental stimuli. Thus, we confirmed that *dhuA* is under nitrogen regulation (27) and that it does not respond to carbon, sulfur, or phosphate availability. A better understanding of the mechanism of regulation of the expression of this operon relative to its control by nitrogen and to other as yet undiscovered aspects requires the isolation and characterization of mutants with alterations of such mechanisms. Such mutants can be obtained by a variety of selective and screening tech-

niques. To determine the nature of these mutations, we developed a rapid way of sequencing numerous independent mutations. The chain termination sequencing method (24) of segments of DNA present on hybrid M13 phages is the method of choice in this case.

The present report describes the isolation of a number of promoter-up mutants and the nucleotide sequences of these mutations. We also describe the in vitro and in vivo construction of a number of M13 hybrid phage derivatives that carry the entire transport operon, a variety of transport mutants, and a method for rapidly transferring the chromosomal promoter-up and other mutations to a hybrid phage for sequencing purposes. In addition, these hybrid phages constitute a set which can be used for picking up any mutation in the transport operon for which there is no positive selection.

MATERIALS AND METHODS

The bacterial and phage strains used or constructed are shown in Table 1. The media used have been described previously (23).

Growth of M13 hybrid derivatives. M13mp2 derivatives that carried the transport clones were maintained and cultured, unless otherwise indicated, in *S. typhimurium* TA3546, which carries a large chromosomal deletion of the entire transport region, thus eliminating the possibility of recombination of the cloned material into the chromosome. An M13 phage culture was obtained either by infecting an early-exponential culture of a bacterial recipient with phage from a single phage plaque or by culturing a bacterial strain that already harbored an M13 hybrid phage carrying the transport operon. The presence of functional transport genes in a phage was determined by infecting strain TA3546 with phage and selecting for D-histidine utilization (DHU) on a minimal salts plus D-histidine plate. Uptake of D-histidine is entirely dependent on a functional transport operon. Phage MA1, which carries the entire 12.4-kilobase-pair (kb) clone, makes very tiny plaques, presumably because of poor reproduction of the very large hybrid phage.

Live-phage electrophoresis. We applied 10^9 to 10^{10} PFU in 10% sucrose plus TE buffer (10 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA) to a vertical 0.8% agarose gel slab (11 by 15 by 0.2 cm) in TAE buffer (40 mM Tris-acetate [pH 8.0], 2 mM EDTA), and electrophoresis was carried out at 50 mA

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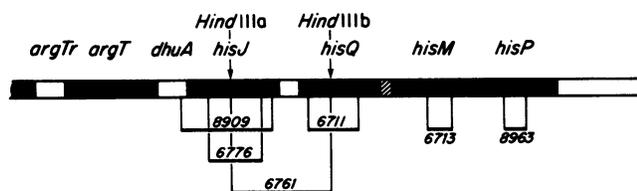


FIG. 1. Schematic representation of the histidine transport operon and of deletions transferred to M13 hybrids. The solid area represents known structural genes. The open areas represent regulatory, terminator, or intergenic regions. The cross-hatched area is a region of ambiguity concerning the start of the *hisM* gene (11). The lines below the chromosome represent deletions that were transferred to M13 hybrids. Deletion $\Delta(hisJQ)6761$ was constructed in vitro by digestion with *Hind*III and ligation.

until bromophenol blue in a control well reached the bottom of the gel (about 1.5 h). Phage bands were stained with 0.005% Coomassie blue in 10% acetic acid and destained in 5% acetic acid. To obtain live phage, we ran a track containing standard phage preparations in parallel with the sample; the portion containing the standard was separated at the end of the run, stained briefly (20 min), destained (5 min), and used as a guide for slicing the unstained portion of the gel in the area where the phage bands were located. Phages M13mp2 and MA1 were used as size standards. The space (3.0 cm) between these two standards (1.4 cm long), as well as the neighboring areas above MA1 and below M13mp2, was sliced in five fragments of ca. 0.6 cm each. Each fragment was placed on a microscope slide and chopped with a razor blade, and the fragments were placed in a small tube containing 0.8 ml of TE buffer. The tubes were left at 4°C overnight with occasional shaking. Recoveries of plaque-forming particles in the buffer were about 50%.

Nucleotide sequence of recombinant phages. Template DNA was prepared by transferring phage and cells present in a single plaque on a lawn of strain TA3546 with a toothpick to 5 ml of nutrient broth and grown at 37°C overnight. The cells were removed by centrifugation in an Eppendorf centrifuge for 5 min; 1.5 ml of the supernatant fraction was added to 200 μ l of 2.5 M NaCl containing 20% polyethylene glycol 6000. After thorough mixing, the solution was left at room temperature for 10 min and then centrifuged for 5 min in an Eppendorf centrifuge. The supernatant fraction was drawn off by aspiration, and the remainder was then centrifuged for 10 s to collect and remove residual supernatant. The small white pellet was suspended in 200 μ l of TE buffer, extracted once with 200 μ l of phenol equilibrated with TE buffer, and then extracted once more with phenol-chloroform (1:1) equilibrated with TE buffer. The aqueous phase was brought to 0.15 M with sodium acetate (pH 4.8), and the DNA was ethanol precipitated, dried, and dissolved in 10 μ l of TE buffer. One microliter contained sufficient DNA to be visible after electrophoresis on a 0.8% agarose gel.

DNA transformation in *S. typhimurium*. Transformation was performed as described previously (18); however, contrary to the previous report, we were unable to achieve efficient transformation (between 10^5 and 10^6 transformants per μ g of DNA) unless a *galE* mutation was present. In addition, in the hope of improving transformability even more, we constructed deep-rough mutants, but these resulted in a loss of transformability for unknown reasons. A *galE* mutation can be introduced into any *Salmonella* strain by transduction with P22 phage prepared on strain TA3425, which carries *galE503* and the linked Tn10 insertion *bio-*

203::Tn10, and by selection for tetracycline resistance (25 μ g/ml) on a tetrazolium-galactose-tetracycline plate (20). *galE* strains appear as red colonies on these plates. It should be noted that when P22 phage is prepared on *galE*-carrying strains, galactose and glucose should be added to the bacterial growth medium. The resulting *bio-203::Tn10 galE503* derivative can either be used as such or transduced to biotin independence and tetracycline sensitivity in a subsequent step with phage cultured on a Bio⁺ strain. The linkage between *bio-203::Tn10* and *galE503* during selection for tetracycline resistance is about 1%.

RESULTS

Multiplication of phage M13 in *S. typhimurium*. Since phage M13 is male specific, it is necessary to construct male *S. typhimurium* strains to be used as hosts. However, such constructs turned out to be very poor hosts. In our initial attempt, we found that M13 (wild type or M13mp2) was not able to yield plaques on a lawn of *S. typhimurium* carrying an F' *lac* factor. Although such a strain could be infected with M13 derivatives that carried *S. typhimurium* cloned material (as evidenced by the recovery of markers), it was not useful for the effective propagation of M13. *S. typhimurium* LT2 carries a high-molecular-weight plasmid known as the "cryptic plasmid," the function of which is unknown (25),

TABLE 1. Bacterial strains and bacteriophages

Strain	Genotype or description
Bacteria^a	
TA831	$\Delta hisF645$
TA3016	$\Delta hisF645 dhuA1 \Delta hisQ6711$
TA3018	$\Delta hisF645 dhuA1 \Delta hisM6713$
TA3180	$\Delta hisF645 dhuA1 \Delta hisJ8909$
TA3258	$\Delta hisF645 dhuA1 \Delta hisP8963$
TA3425	<i>galE503 bio-203::Tn10</i>
TA3546	$\Delta hisF645 \Delta(his-ubiX-dhuA-hisJQMP-ack-pta)6745/F'42 lac^+ fin-301$
TA3587	<i>recA1 \Delta hisF645</i>
TA3592	$\Delta hisF645 dhuA1 \Delta hisJ6776$
TA3600	TA3546 also carrying <i>galE503</i>
TA3631	$\Delta hisF645 dhuA509$
TA3632	$\Delta hisF645 dhuA510$
TA3633	$\Delta hisF645 dhuA511$
TA3634	$\Delta hisF645 dhuA512$
SA2197 ^b	<i>purC7/F'42 lac^+ fin-301</i>
SA2201 ^b	<i>purC7/F'42 lac^+</i>
Phage	
M13mp2 ^c	M13 carrying a portion of the <i>lac</i> operon
MA1 ^d	M13mp2 hybrid carrying 12.4 kb of <i>S. typhimurium</i> DNA
MA2→MA9	In vivo deletions of the nontransport region in MA1
MA10 ^e	In vitro deletion $\Delta(hisJQ)6761$ by <i>Hind</i> III restriction digest of MA4
MA11	Derivative of MA10 carrying $\Delta hisJ6776$
MA12	Derivative of MA10 carrying $\Delta hisQ6711$
MA13	Derivative of MA10 carrying $\Delta hisM6713$
MA14	Derivative of MA10 carrying $\Delta hisP8963$
MA16	Derivative of MA10 carrying $\Delta hisJ8909$

^a Minimal and rich media have been described previously (23).

^b Kindly supplied by K. Sanderson. The F' *lac*⁺ plasmid present in strain SA2197 was transferred into any *S. typhimurium* strain by selecting for lactose utilization and counterselecting against the purine requirement. As a control, the F' *lac*⁺ plasmid present in strain SA2201 was also transferred into one of the strains. Only derivatives carrying the *fin-301* mutation plaque M13 phage.

^c Kindly supplied by J. Messing (8).

^d Described previously (2).

^e DNA transformed into strain TA3600.

and which apparently interferes with maleness functions. Therefore, strains which had been cured of the cryptic plasmid (constructed and supplied by H. Whitfield) were rendered male by introduction of an $F' lac$ plasmid and were able to support M13 phage growth effectively. However, such strains grew more poorly than cryptic plasmid-containing strains on poor carbon sources and acquired spontaneous mutations at a very high frequency, which restored good bacterial growth but concomitantly caused a loss in the ability to plaque phage M13 (data not shown). It is not known why cryptic plasmid-cured strains grow poorly if rendered male and what mutation(s) is responsible for restoring their growth ability. It seems that a deletion of a portion of the $F' lac$ plasmid might be involved since the Lac^+ phenotype was lost simultaneously with the restoration of good growth and loss of ability to plaque M13. An alternative solution was found. It has been suggested (K. E. Sanderson, personal communication) that F factors that carry mutations in the *fin* episomal gene might be able to overcome repression by the cryptic plasmid. Indeed, the introduction of $F'42 lac^+ fin-301$ (7) into any *S. typhimurium* strain allows effective infection and growth of M13 phage (the usual yield is about 10^{12} phage per ml). When it was necessary to introduce M13 or its derivatives into *S. typhimurium*, a male derivative carrying $F'42 fin-301$ was always constructed first.

Selection of smaller hybrid phage derivatives. A hybrid M13 phage (MA1) that carries the entire histidine transport operon and the neighboring *argT* monocistronic operon (Fig. 1) was utilized for the construction of derivatives useful for the transfer of chromosomal mutations (2). The presence of the large cloned fragment of DNA (12.4 kb) resulted in a recombinant phage of very high instability. Growth of MA1 on strain TA3546 in rich, unselective medium yielded 4×10^{12} phage per ml, of which only 0.1% still carried the inserted DNA. However, growth in minimal salts medium in the presence of 10^{-4} M D-histidine (the uptake of which is entirely dependent on a functional histidine transport operon) yielded between 10^{10} and 10^{11} phage per ml, of which from 10 to 100% still contained the transport operon. Because of this instability of MA1, it was necessary to select for smaller derivatives, which resulted in higher stability. We speculated that in any phage population there might be defective phage particles that have lost fragments of DNA from the inserted DNA fragment and/or from the phage DNA proper. Such phage particles would be smaller than the starting MA1 phage because the size of M13 phage is proportional to the amount of DNA it contains (12). Thus, it should be possible to separate these smaller phages by size (4) by using live-phage electrophoresis on an agarose gel. Figure 2 shows the results of a separation performed on phage cultured on cells grown in rich medium, i.e., without selective pressure to retain the transport operon. Similar results, although with different proportions among the various phages, were obtained also from phage grown on cells under selective pressure (minimal medium with D-histidine as the histidine source). Two classes of plaques could be differentiated, roughly grouped as large and small (Fig. 2). The large plaques were presumably caused by either M13mp2 arising by loss of the entire cloned material or by derivatives of it carrying much smaller fragments of the cloned DNA. Large plaque-yielding phages were most abundant in section 5, which is the region to where M13mp2 migrated, and, upon testing, did not impart DHU ability to strain TA3546 (see legend to Fig. 2). The bulk of the small plaque-yielding phages was found in section 3, where MA1

migrated. The class of interest is the one that imparted DHU and migrated to sections 4 and 5, which presumably included phages smaller than MA1. Section 4 had a higher ratio of DHU phage to PFU than did section 3, possibly an indication of the increased stability of smaller DHU phages as compared with MA1. Thirty-six DHU colonies infected with phage originating from sections 3 to 5 from several experiments were purified once on D-histidine, and the size of phage DNA present in these single colonies was determined. Five colonies yielded DNA that was clearly smaller than MA1 DNA. By repeated subculture and electrophoresis, the following independent derivatives were obtained and characterized by restriction mapping (Fig. 3): MA2, MA4, MA5, MA6, and MA8. MA6 seems to be identical to MA4, although each certainly arose independently. Several additional, apparently identical phages were discarded because their independent origins could not be established. However, this might indicate the existence of favorite sites for deletion ends. Figure 3 shows the genealogy of these phages and the physical map of their DNAs. Because these phages were screened for and grown under selective pressure to maintain the transport genes, all contained an intact histidine transport operon. Two of the deletions (in MA4 and MA6) extended into the *argT* gene, which codes for an additional, but not essential, transport component the lysine-, arginine-,

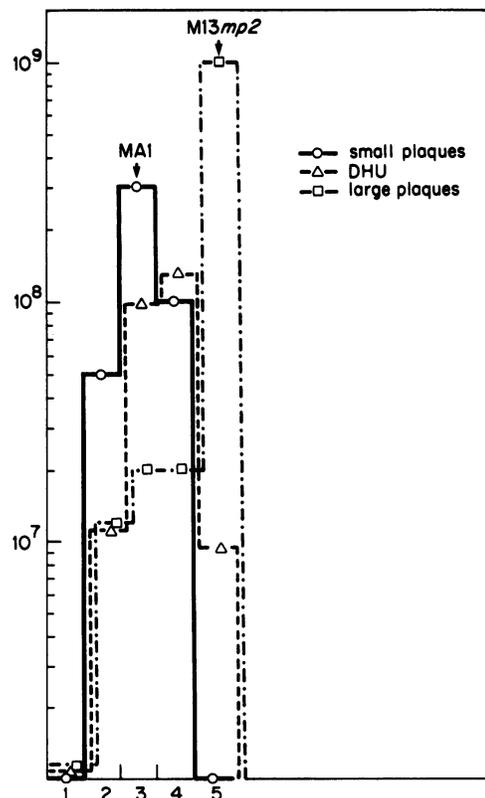


FIG. 2. Live-phage electrophoresis. MA1 phage was electrophoresed as described in the text. The eluate from each section (numbered as indicated on the abscissa) was assayed for the presence of PFU and of phage able to impart DHU ability (ordinate). Symbols: ○, small plaques; □, large plaques; △, DHU phage. DHU ability was determined by infecting strain TA3546 with the phage and selecting for growth on D-histidine. The two arrows at the top indicate the locations of the MA1 and M13mp2 phages on a parallel portion of this separation stained with Coomassie blue.

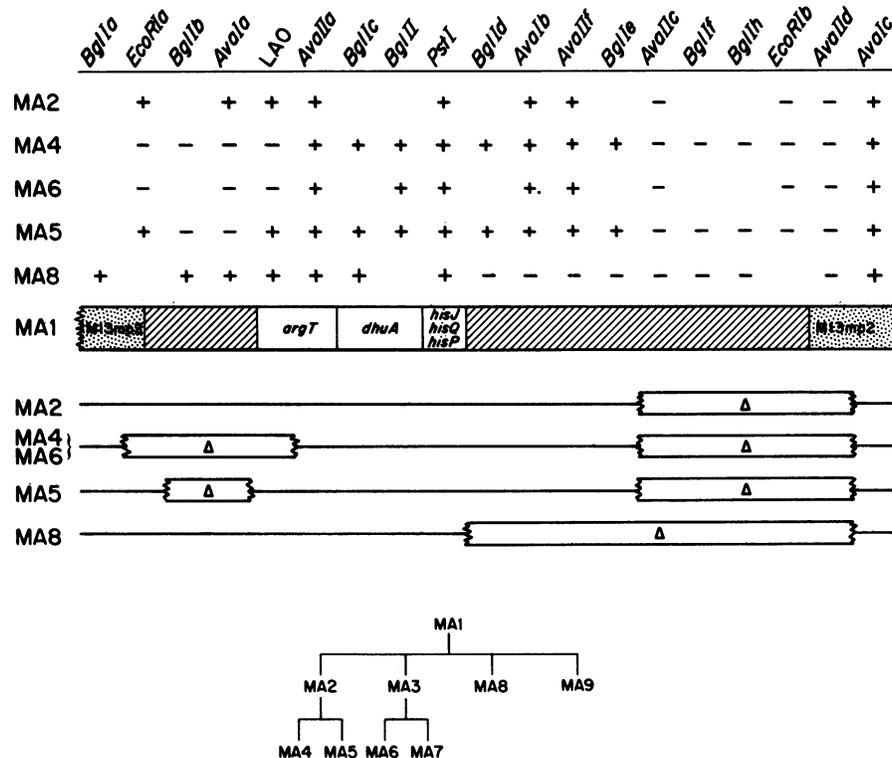


FIG. 3. Restriction maps of MA1 derivatives. The schematic representation of MA1 is not to scale; it is meant to emphasize restriction sites. Regions: M13mp2 material; *lac* operon material plus cloned DNA other than the transport operon; transport operon. Restriction maps of MA3, MA7, and MA9 phages were apparently identical to those of MA2, MA6, and MA8, respectively. Since the nucleotide sequences at the deletion ends are not known, the phages may actually differ slightly (and they have been saved). The genealogy of the phages is shown at the bottom. The Rf form of the phage DNA was prepared by a standard clear-lysate procedure and purified by sedimentation in CsCl.

ornithine-binding protein. This was established by determining the presence of this protein by sodium dodecyl sulfate-acrylamide gel electrophoresis of shock fluids from strain TA3546 cells infected with each of these phages (data not shown) (15). Thus, phages that are smaller by 4 (MA2) to 7.5 kb (MA4 and MA6) of DNA and that are much more stable during growth under a variety of conditions can be obtained by this selection. Some of these have been used to create additional derivatives to be used for the nucleotide sequencing of mutations of interest as shown below.

Transfer of deletion mutations to hybrid phage. To transfer promoter-up and other interesting transport mutations onto the phage for sequencing purposes, it was necessary to have an initial collection of transport-negative mutations to be used for infecting complementary mutants of interest, thus allowing their transfer onto the phage by recombination during growth under selective conditions. The method devised, similar to the one described by Bossi and Roth (6), requires the initial presence on the phage of a nonreverting mutation. An *in vitro* deletion [Δ (*hisJQ*6761)] was constructed in phage MA4 by digestion with *Hind*III endonuclease (which removes a 0.75-kb fragment of DNA), followed by recircularization with T4 DNA ligase. The DNA was then introduced into a *galE* derivative (TA3600) by transformation (Table 1). The phage obtained, named MA10, was used to build several deletion mutant recombinants *in vivo* using well-characterized operon deletions. Phage MA10 was used to infect male strains, each of which carried a deletion of interest and which selected for complementation to DHU

ability. In each case, phage produced from several DHU colonies was tested for its ability to complement two different strains to DHU ability: (i) the initial deletion recipient strain and (ii) strain TA3592 (Δ *hisJ6776*). Only phages able to complement Δ *hisJ6776* (MA10 cannot), but not the initial deletion recipient strain, carried the desired mutation. These were purified by single-plaque isolation and culturing on TA3546. When the deletion to be transferred did not complement MA10, the first step involved infection and growth on nonselective rich medium. The rare recombinant phages carrying the desired deletion were selected by their ability to complement an appropriately chosen chromosomal deletion that MA10 cannot complement; e.g., the recombination of Δ *hisJ8909* into MA10 (to create MA16) was screened for by complementation of Δ *hisQ6711*, which the original MA10 cannot complement. Deletions Δ *hisJ6776*, Δ *hisJ8909*, Δ *hisQ6711*, Δ *hisM6713*, and Δ *hisP8963* were thus transferred to MA10 (Fig. 1). Each of the mutations transferred to MA10 was rechecked by mapping by standard techniques and named (Table 1). These phages constitute a set that can now be used as the starting material for picking up any mutation in the operon. They were used for picking up *dhua* mutations as shown below. They were also used for picking up and sequencing an interesting class of *hisM* mutations, the "odd group" (1; Payne and Ames, unpublished data).

Selection and sequence of promoter-up mutations. Promoter-up mutants were selected on the basis of the improved utilization of D-histidine, the transport of which through the histidine transport system is the limiting step in its utiliza-

nitrogen regulation. The methods we presented which allow the transfer of chromosomal mutations to M13 hybrid derivatives and efficient reproduction of M13 in *S. typhimurium* should greatly simplify the systematic analysis of additional mutations and be useful for similar studies in other systems.

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