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# *Salmonella typhimurium* Histidine Periplasmic Permease Mutations That Allow Transport in the Absence of Histidine-Binding Proteins

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**Periplasmic transport systems consist of a membrane-bound complex and a periplasmic substrate-binding protein and are postulated to function by translocating the substrate either through a nonspecific pore or through specific binding sites located in the membrane complex. We have isolated mutants carrying mutations in one of the membrane-bound components of the histidine permease of *Salmonella typhimurium* that allow transport in the absence of both histidine-binding proteins HisJ and LAO (lysine-, arginine-, ornithine-binding protein). All of the mutations are located in a limited region of the nucleotide-binding component of the histidine permease, HisP. The mutants transported substrate in the absence of binding proteins only when the membrane-bound complex was produced in large amounts. At low (chromosomal) levels, the mutant complex was unable to transport substrate in the absence of binding proteins but transported it efficiently in the presence of HisJ. The alterations responsible for the mutations were identified by DNA sequencing; they are closely related to a group of *hisP* mutations isolated as suppressors of HisJ interaction mutations (G. F.-L. Ames and E. N. Spudich, Proc. Natl. Acad. Sci. USA 73:1877–1881, 1976). The *hisP* suppressor mutations behaved similarly to these newly isolated mutations despite the entirely different selection procedure. The results are consistent with the HisP protein carrying or contributing to the existence of a substrate-binding site that can be mutated to function in the absence of a binding protein.**

Periplasmic transport systems (permeases) in gram-negative bacteria have a common organization, usually consisting of one or more periplasmic substrate-binding receptor proteins and three to four membrane-bound proteins (reviewed in references 2 and 5). These permeases transport a wide variety of substrates with high affinity despite their very similar overall composition. The components that impart specificity are hydrophilic proteins ranging in molecular mass from 22,0000 to 56,000 Da that bind the substrate with high affinity and are usually present in the periplasm in high concentration. Two of the membrane components have hydrophobic sequences and are usually assumed to be integral membrane proteins. The other membrane protein(s) has an obviously hydrophilic sequence (11, 12), despite its being found in association with the membrane, and is usually thought of as a peripheral membrane protein. In comparisons between different permeases, extensive sequence conservation is found in the latter membrane component, with two regions of particularly high similarity centering around two motifs implicated in nucleotide binding (4a; reviewed in references 2 and 13).

The histidine permease of *Salmonella typhimurium* is among the best characterized of the periplasmic transport systems. It consists of a histidine-binding protein, HisJ, two hydrophobic membrane proteins, HisQ and HisM, and the nucleotide-binding membrane protein HisP. HisQ and HisM have been shown by a variety of methods to span the membrane; the three membrane-bound proteins form a complex (Q/M/P), as shown by cross-linking and coimmunoprecipitation experiments (15a). The histidine-binding protein HisJ has been shown to interact directly with HisQ (24) and possibly with HisP (8). In addition, a second periplasmic protein, the lysine-, arginine-, ornithine-binding protein (LAO), transports these amino acids in combination with the Q/M/P complex; LAO also binds and transports histidine with poorer affinity. These permeases are energized by ATP hydrolysis (reviewed in reference 3). HisP has been shown to bind nucleotides (14), and its nucleotide-binding site has been characterized (20, 21). Therefore, HisP may be the subunit responsible for coupling ATP hydrolysis to transport.

A model for periplasmic transport envisions an initial interaction between the substrate and the binding protein, resulting in the formation of the actual transport substrate, the liganded binding protein. Once the binding protein is liganded, it is postulated to interact preferentially with the membrane-bound complex, which undergoes conformational changes, allowing the formation either of a pore through which the substrate diffuses or, alternatively, of specific substrate-binding sites to which the substrate is transferred on its way through the membrane. The powerful concentrative action of periplasmic permeases has recently been clarified to be energized by ATP (6, 9, 10, 15, 23; reviewed in reference 3). Experimental support for the binding-site model requires the demonstration of substrate-binding activity by the membrane component(s). Such activity has never been demonstrated. However, indirect genetic evidence supporting the binding-site model has been obtained through mutations located in the hydrophobic membrane components of the histidine permease, which alter substrate transport specificity (22), and through several mutations in the hydrophobic components of the maltose permease, which allow transport in the absence of the respective binding protein (27).

The possibility that HisP might also carry a substrate-binding site arose because of genetic evidence that HisP interacts directly with HisJ. A *hisJ* mutation (*hisJ5625*),

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TABLE 1. Strains

| Chromosome genotype[a] | Strain with plasmid[b]: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | None | pGW7 | pFA53 (none) | pFA108 (*hisP9085*) | pFA109 (*hisP9086*) | pFA110 (*hisP9087*) | pFA111 (*hisP9088*) | pFA123 (*hisP9016*) | pFA124 (*hisP9083*) |
| Δ*hisF645 dhuA1* | TA271 | TA4185 | | | | | | | |
| *his*$^+$ *dhuA1 ntrB139* | TA3290 | | | | | | | | |
| Δ*hisF645 dhuA1* Δ*hisJ6776 hisJ9084 argT527* (Mu dI) | GA19[c] | | | | | | | | |
| Δ*hisF645 dhuA1* Δ*his(AJQMP argT ubiX)6631* | TA3162 | | GA21 (GA85) | GA22 (GA86) | GA23 (GA87) | GA24 (GA88) | GA25 (GA89) | GA143 | GA144 |
| Δ*hisF645 dhuA1 hisJ5625* | TA308 | | GA80 | GA81 | GA82 | GA83 | GA84 | | |
| Δ*hisF645 dhuA1* Δ*his(QMP)5575* | TA1741 | | GA96 | GA97 | GA98 | GA99 | GA100 | GA145 | GA146 |
| *his*$^+$ *dhuA1* Δ*his(QMP)5575* | TA1871 | | | | | | | | |
| Δ*hisF645 dhuA1* Δ*his(MP)5549* | TA1708 | | GA101 | GA102 | GA103 | GA104 | GA105 | | |
| Δ*hisF645* Δ*his(AJQMP argT ubiX)6631 zig-205*::Tn*10 ntrB139* | | | GA106 | GA107 | GA108 | GA109 | GA110 | | |
| Δ*hisF645 dhuA1* Δ*hisJ6776* | TA2918 | | TA4181 | GA112 | GA113 | GA114 | GA115 | | |
| Δ*hisF645 dhuA1* Δ*his(JQMP) 6572 zig-205*::Tn*10 ntrB139* | GA151 | | GA152 | GA153 | GA154 | GA155 | GA156 | GA157 | GA158 |

[a] *dhuA* is the promoter of the histidine transport operon, and *dhuA1* is a constitutive mutation; *hisF* is a gene of the histidine biosynthetic operon; *ntrB139* is a nitrogen regulatory mutation elevating expression of proteins involved in nitrogen utilization.

[b] pGW7 is the vector plasmid from which pFA53 was derived and thus it carries no transport genes. The other plasmids carry the mutations indicated. Strains in parentheses are *his*$^+$ derivatives.

[c] Mutation *hisJ9084* is a duplication resulting in a fusion of the amino-terminal one-third of HisJ to the carboxy-terminal two-thirds of LAO (26a).

resulting in an altered HisJ with intact histidine-binding activity but unable to interact with the membrane complex and thus mutated in its interaction site (16), was found to be suppressible by mutations in HisP (8, 22a, 26). This evidence that HisP comes into direct contact with HisJ suggests that domains of HisP are close to the periplasmic surface and thus may also come in contact with the substrate. While there are other possible explanations for the genetic suppression results (such as a cascade of interactions via HisQ and HisM), the possibility exists that HisP receives the substrate directly from the binding protein. Therefore, evidence that HisP can function in the absence of binding protein(s) was sought as an indication that it may carry a substrate-binding site.

In this article, we describe the isolation and analysis of a set of *hisP* mutants that can transport substrate in the complete absence of both HisJ and LAO, the two periplasmic binding proteins essential for histidine transport through this permease.

## MATERIALS AND METHODS

**Strains and media.** Bacterial strains are listed in Table 1. The following media were used: minimal medium E with 0.4% glucose as the carbon and energy source (23); and LB medium (19); minimal medium without added carbon or nitrogen source ($N^-C^-$ [17]). Plasmid-containing strains were grown in the presence of ampicillin (100 μg/ml). In addition, all strains carrying Δ*his-6631* (which deletes *ubiX*, involved in ubiquinone biosynthesis) were supplemented with *p*-hydroxybenzoic acid (10 μg/ml) when grown in minimal medium. Phage P22 transductions were performed as described before (25), with P22 HT*int-4* phage for all manipulations. All routine genetic manipulations and maintenance of plasmid-carrying strains were performed at 30°C unless otherwise stated.

**DES mutagenesis.** An 0.05-ml amount of a stationary-phase culture of GA21 in LB medium was diluted in 5 ml of medium E; 3 drops of diethylsulfate (DES) were added, and the mixture was vortexed for 1 s. After the DES was allowed to settle for 20 min at room temperature, 0.5-ml aliquots of the mutagenized culture were added to individual tubes containing 5 ml of LB-ampicillin medium and grown to saturation. Mutants obtained from each of these final cultures were considered independent.

**Selection of D-histidine-utilizing derivatives of GA21.** A 0.1-ml amount of a DES-mutagenized GA21 culture was placed on minimal medium E plates containing 300 μM D-histidine and incubated at 37°C. Ten independent cultures from each of two separate mutagenesis procedures were used. Each plate gave rise to more than 50 small colonies after 48 h of incubation, and four colonies of various sizes were picked from each plate and purified at 30°C, and their D-histidine growth ability was measured at 37°C by radial streak (7).

**L-Histidine transport assays.** Transport in histidine prototrophs was measured by the protein incorporation assay (1). Transport in histidine auxotrophs was assayed by washed-cell uptake assays as follows. Cells were grown to exponential phase in medium E containing glucose, ampicillin, and $10^{-4}$ M histidine, then chilled on ice, and harvested at 8,000 × *g* for 15 min at 4°C. The cells were washed twice with medium E at 0°C and then resuspended in the same growth medium in the absence of histidine. The cells were incubated at 37°C for 10 min with aeration before initiating the assay to ensure the depletion of residual histidine. The assay was initiated by the addition of L-[$^3$H]histidine (New England Nuclear). At 30-s or 1-min intervals, 0.5-ml samples were withdrawn, filtered on glass fiber filters (Schleicher & Schuell), and washed with 7.5 ml of medium E. Dried filters were counted with Scint-A liquid scintillation cocktail in a Searle Delta 300 scintillation counter. Counts per minute were converted to picomoles per milligram (dry weight) by using a conversion factor of 47 μg (dry weight) of cells per ml per $OD_{650}$ unit.

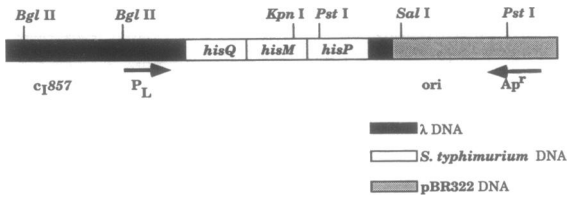


FIG. 1. Linearized restriction map of plasmid pFA53. Genes *hisQ*, *hisM*, and *hisP* (open boxes) are placed under the control of the lambda $p_L$ promoter. Dark boxes, Lambda DNA; stippled boxes, pBR322 DNA.

**Recombinant DNA.** Recombinant DNA procedures were performed as described before (18). Purification of restriction fragments from agarose gels was done with Gene Clean (Bio101, La Jolla, Calif.). Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs or Boehringer Mannheim Biochemicals. pGW7 was obtained from M. J. Chamberlin. Plasmids were transferred between strains by phage P22 transduction, selecting for ampicillin resistance.

**Construction of pFA53.** In order to overproduce HisQ, HisM, and HisP in *S. typhimurium*, the genes encoding them were placed under the control of the phage lambda $p_L$ promoter and the *c*I857 temperature-sensitive repressor that are present in plasmid pGW7. pFA17 (which carries the transport genes under the control of the lambda $p_L$ promoter [14] and shares all the lambda sequences of pGW7, but does not carry the *c*I857 repressor gene) was used as the source of DNA carrying the transport genes and the $p_L$ promoter. A *Bam*HI-*Bgl*II fragment from pFA17 containing the $p_L$ promoter and the transport genes was ligated into pGW7, replacing the equivalent lambda sequences, and transformed into *Escherichia coli* 294, with selection for Amp$^r$ at 30°C. The resulting plasmid was denoted pFA53 (Fig. 1). Strains carrying pFA53 and pGW7 were grown to stationary phase in LB-ampicillin at 30 or 37°C. Whole-cell proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with antibodies raised against HisQ and HisP. Strains carrying pFA53 and a chromosomal deletion of *hisQ*, *hisM*, and *hisP* produced elevated levels of HisQ and HisP at 37 but not at 30°C. Strains carrying pGW7 did not produce these proteins at either temperature. pFA53 was transferred from *E. coli* into the restriction-modification-defective *S. typhimurium* strain SL4213.

**Mapping of plasmid mutations.** Plasmid DNA isolated from pFA53 and from each of the mutants was digested with *Kpn*I and *Sal*I (see Fig. 1 for restriction map), and the *hisM*- and *hisP*-containing fragment of pFA53 was replaced with the corresponding fragment from each mutant so as to reconstruct the operon. The resulting plasmids were retransformed into SL4213 and then transduced into TA3162.

**Transfer of *hisP9016* and *hisP9083* from the chromosome to pFA53.** Chromosomal DNA from strains carrying either *hisP9016* or *hisP9083* were amplified by polymerase chain reaction with the sequences flanking and carrying sites for *Pst*I (in the 5′ portion of *hisP*) and *Stu*I (downstream from the 3′ end of *hisP*) as primers (26). The amplified products were digested with *Pst*I and *Stu*I, then ligated into pFA53 which had been digested with *Sal*I, blunted with Klenow, and digested with *Pst*I. The resulting plasmids were transformed into *E. coli* 294, their DNA sequence was confirmed, and they were subsequently transferred into *S. typhimurium*.

## RESULTS

**Isolation of mutants able to transport D-histidine in the absence of periplasmic histidine-binding proteins.** A strain was constructed that lacked the chromosomal genes *hisJ* and *argT*, which code for the two periplasmic proteins HisJ and LAO, respectively, known to bind histidine and to utilize Q/M/P to function. In order to facilitate genetic and recombinant DNA manipulations, the genes coding for the membrane-bound transport components were placed on a plasmid (pFA53) and introduced into a histidine auxotrophic strain (TA3162) carrying a chromosomal mutation deleting the entire histidine transport operon and the *argT* gene, yielding strain GA21. Since growth on D-histidine as a source of L-histidine is entirely dependent on an intact histidine permease (1), GA21 is unable to grow on D-histidine at any temperature. However, if the permease membrane proteins could be mutated to transport D-histidine in the absence of periplasmic proteins, growth of such mutants should occur at 37 but not at 30°C because of the temperature-sensitive expression of the transport genes in pFA53.

GA21 was mutagenized and plated on ampicillin- and D-histidine-containing plates at 37°C. Colonies appearing after 48 h of incubation were screened for their ability to grow on D-histidine at 37 but not at 30°C. In order to determine whether mutations responsible for this pattern of growth were due to alterations in the membrane permease complex and independent of additional chromosomal mutations, plasmids from each of these strains were transferred by phage P22 transduction into TA3162, and the Amp$^r$ transductants were tested for D-histidine growth at 37°C. Seven independent mutants were determined to contain exclusively plasmid-carried, D-histidine-utilizing mutations: GA22, GA23, GA24, D8-1, GA25, E6-1, and E8-1. The growth of the mutants was tested by the radial streak test; all grew at 37°C to a radius of 5 to 10 mm, compared with 16 mm for a control strain with an intact transport operon (TA271). The mutants grew more poorly at 33°C, and none grew at all at 30°C (data not shown). These data show that the ability to grow on D-histidine is dependent on the elevated expression of the mutant membrane proteins and that the lower level of these proteins produced at 33°C is limiting for growth. These mutants are likely to carry an alteration(s) in one of the membrane protein-encoding genes present on pFA53, in such a way that the resulting permease complex functions in the absence of both HisJ and LAO. Strains D8-1, E6-1, and E8-1 were found to be identical to GA23 and were not used further.

**Growth characteristics of the mutants.** The ability of the mutants to grow on D-histidine and other transport substrates was quantitated and compared with that of a wild-type strain and of the parent (Table 2). At 3 mM D-histidine, GA22 to GA25 grew with a doubling time twice that of TA271. Higher D-histidine concentrations did not improve growth (data not shown). Growth on carnosine, which is not dependent on the histidine permease, and on L-histidine, which has several other means of entry into the cell (7), was unaffected. An estimate of the affinities of the mutant permeases for D-histidine was obtained by measuring growth on D-histidine concentrations ranging from 100 μM to 5 mM. The apparent affinity constants were between 450 and 700 μm, at least 50-fold higher than that of TA271 (Table 2, last column). The parental strain GA21 did not grow on D-histidine at any concentration or temperature.

Growth on two other substrates of the histidine permease, *N*-acetyl-L-histidine and arginine, was also tested by radial

TABLE 2. Growth of plasmid mutants on various histidine sources

| Strain | Plasmid | Doubling time (min)[a] | | | | $K_m$ for D-histidine[b] (μM) |
|---|---|---|---|---|---|---|
| | | Carnosine, 37°C | L-Histidine, 37°C | D-Histidine 30°C | D-Histidine 37°C | |
| GA22 | pFA108 (*hisP9085*) | 64 | 84 | NG | 99 | 500 |
| GA23 | pFA109 (*hisP9086*) | 68 | 84 | NG | 105 | 700 |
| GA24 | pFA110 (*hisP9087*) | 74 | 80 | NG | 104 | 450 |
| GA25 | pFA111 (*hisP9088*) | 64 | 78 | NG | 101 | 500 |
| GA21 | pFA53 (wild type) | 60 | 60 | NG | NG | NA |
| TA271 | | 51 | 51 | 60 | 51 | <10 |

[a] Growth curves were performed in medium E-glucose-ampicillin-*p*-hydroxybenzoic acid with 300 μM histidine source, except for D-histidine, which was used at 3 mM. NG, No growth.

[b] The $K_m$ for D-histidine uptake was calculated from the doubling times obtained on various concentrations of D-histidine. NA, Not applicable.

streaks (Table 3). GA21 had a low level of *N*-acetyl-L-histidine growth, and the mutants did not show any improvement. Growth on arginine as a source of nitrogen is dependent on elevated levels of LAO and of the membrane-bound complex of the histidine permease (17). GA21 was unable to grow on arginine as a nitrogen source, and again the mutants showed no improvement. This result was essentially the same in strains GA106 through GA110, which carry the nitrogen regulatory mutation *ntrB139*, which constitutively elevates the levels of histidine and arginine transport components and of the nitrogen assimilation enzymes (17). The low level of growth on arginine of GA106 was much poorer than that of an *ntrB139* strain with a wild-type permease (TA3290) and was unchanged in the presence of the plasmid mutations. These data indicate that the mutant transport complexes are unable to recognize *N*-acetyl-L-histidine and arginine.

As an alternative to growth measurements, the affinity of the mutant transport systems for arginine and lysine (another LAO substrate) was also measured by their ability to inhibit growth on D-histidine through their competition with D-histidine transport (4). The inhibition of growth by 10 μmol of L-arginine or L-lysine on a petri plate containing 2 mM D-histidine was tested by radial streaks. The growth of all the mutants was unaffected, while TA271 and GA19 (a strain that utilizes a chimeric fusion protein composed of portions of HisJ and LAO that binds arginine) displayed an inhibition zone with a radius of 22 and 24 mm with arginine and 19 and 21 mm with lysine, respectively (data not shown). Thus, the mutants neither recognize nor transport *N*-acetyl-histidine, arginine, or lysine. These results indicate that the mutations have resulted in a membrane complex that is competent to transport substrate without the participation of the known specificity-bearing determinants, the binding proteins, and that effects transport with a different pattern of substrate specificity than that of the wild-type permease.

TABLE 3. Growth of plasmid mutants on L-arginine and *N*-acetyl-L-histidine

| Strain | Plasmid | *ntrB*[a] | Growth[b] | | |
|---|---|---|---|---|---|
| | | | Arginine | *N*-Acetyl-L-histidine | D-Histidine |
| GA21 | pFA53 (wild type) | + | − | +/− | − |
| GA22 | pFA108 (*hisP9085*) | + | − | +/− | + |
| GA23 | pFA109 (*hisP9086*) | + | − | +/− | + |
| GA24 | pFA110 (*hisP9087*) | + | − | +/− | + |
| GA25 | pFA111 (*hisP9088*) | + | − | +/− | + |
| GA106 | pFA53 (wild type) | − | +/− | +/− | − |
| GA107 | pFA108 (*hisP9085*) | − | +/− | +/− | + |
| GA108 | pFA109 (*hisP9086*) | − | +/− | +/− | + |
| GA109 | pFA110 (*hisP9087*) | − | +/− | +/− | + |
| GA110 | pFA111 (*hisP9088*) | − | +/− | +/− | + |
| TA3290 | | − | ++ | ND | ND |
| TA271 | | + | ND | ++ | ++ |

[a] *ntrB* is either wild type (+) or the *ntrB139* mutation (−).

[b] Growth was measured at 37°C by radial streak with: for arginine, $N^-C^-$/glucose medium and a disk containing 10 μmol of L-arginine as the nitrogen source; for D-histidine and *N*-acetyl-L-histidine, medium E and disks containing 1 and 0.2 μmol, respectively, of D-histidine and *N*-acetyl-L-histidine. Plates were incubated for 48 h. ND, Not determined. Symbols: −, no growth; +/−, weak growth; +, growth; ++, strong growth.

The possibility that the mutated membrane complex utilizes an unknown binding protein with a different spectrum of specificity was explored by testing each mutant for inhibition of growth on D-histidine by a variety of compounds as a broader measure of substrate specificity. The following compounds were tested for growth inhibition by placing them, in groups of four or five, on minimal medium plates containing 2 mM D-histidine: all L-amino acids plus glycine, biotin, thiamine, pyridoxal, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, lipoic acid, pantothenate, diaminopimelic acid, vitamin $B_{12}$, nicotinic acid, adenine, guanine, cytosine, uracil, thymine, D-tyrosine, D-phenylalanine, D-serine, D-threonine, D-arginine, D-lysine, D-aspartate, D-alanine, D-glutamate, maltose, arabinose, galactose, ribose, fructose, alanyl-glycine, and arginyl-glycyl-glycine. None of these compounds inhibited growth (except adenine, which also inhibited growth on L-histidine) (data not shown). The failure of all of these substances to compete with D-histidine demonstrates that none of them is a substrate of the mutated permeases, and it implies that the mutants are not making use of another periplasmic binding protein, since it would have been likely to be one responsible for the transport of one of these substances.

**Measurement of transport.** Growth of the mutants on D-histidine demonstrates that they do transport this substrate. In order to quantitate uptake activity through Q/M/P, we assayed L-histidine transport. None of the mutants showed a significant increase in transport over the parental strain, GA21 (Table 4). It should be noted that GA21 had no increase in transport over two control strains, TA3162 and TA1871, both of which are deleted for chromosomal *hisQ*, *hisM*, and *hisP* and carry no plasmid. The wild-type strain TA271 exhibited the expected high level of transport. A more accurate assay of transport was performed by the protein incorporation method to obtain an apparent $K_m$ value, using strains prototrophic for histidine (GA85 to GA89). By this assay, the apparent $K_m$ of transport for the mutants was about 3 μM and not significantly different from that of GA21 (data not shown). Since any mutation that restores growth on D-histidine would also be expected to increase L-histidine transport, the most likely explanation is that the residual L-histidine transport activity in GA21, which is due to other known histidine permeases (7), masks any newly arisen but weak L-histidine transport activity. The $K_m$ measurements were repeated in the presence of aromatic amino acids, with no change in the activity of either the parental or the mutant strains. This indicates that the aromatic permease (7) does not contribute a significant fraction of this residual transport activity at the histidine concentrations used. To determine whether this residual transport activity in GA21 and in mutants GA22 through GA25 was due to the action of a periplasmic permease, all strains were

TABLE 4. L-Histidine transport by plasmid mutants

| Strain | Relevant genotype | Plasmid | Transport of L-histidine[a] (pmol/min per mg [dry wt]) |
|---|---|---|---|
| TA3162 | *Δhis(JQMP argT ubiX)6631* | None | 1.5 |
| TA1871 | *Δhis(QMP)5575* | None | 1.5 |
| TA271 | | None | 384 |
| GA21 | *Δhis(JQMP argT ubiX)6631* | pFA53 (wild type) | 1.7 |
| GA22 | *Δhis(JQMP argT ubiX)6631* | pFA108 (*hisP9085*) | 1.8 |
| GA23 | *Δhis(JQMP argT ubiX)6631* | pFA109 (*hisP9086*) | 1.6 |
| GA24 | *Δhis(JQMP argT ubiX)6631* | pFA110 (*hisP9087*) | 1.9 |
| GA25 | *Δhis(JQMP argT ubiX)6631* | pFA111 (*hisP9088*) | 1.6 |

[a] Assayed by the washed-cell uptake method with 20 nM L-[$^3$H]histidine.

subjected to osmotic shock: in all cases, residual transport activity was insensitive to osmotic shock (data not shown), confirming that it is due to systems that do not utilize periplasmic components.

**Mapping and sequencing the mutations.** The plasmid fragment responsible for the mutant phenotype was identified by replacing restriction fragments of plasmid pFA53 with equivalent restriction fragments purified from each mutant plasmid and then testing the ability of the resulting recombinant plasmids to impart D-histidine growth when placed into TA3162. All seven mutations were located on the *Kpn*I-*Sal*I restriction fragment (Fig. 1) containing the 3′ portion of *hisM* and all of *hisP*. The converse experiment, in which a wild-type restriction fragment from pFA53 was used to replace the mutated region in each of the seven mutant plasmids, resulted in loss of D-histidine growth. By a similar approach, the location of the mutations was narrowed down to the smaller *Pst*I-*Sal*I fragment that contains only a portion of *hisP*. The *Kpn*I-*Sal*I fragment was cloned into M13mp18, and the nucleotide sequence was determined from the *Pst*I site to the *hisP* stop codon. The sequence changes in the four mutations lay within 100 bp of each other. The residue changes are shown in Fig. 2. Two mutations, *hisP9085* and *hisP9088*, affected the same codon. We noted that several *hisP* suppressor mutations also lay in this region, *hisP5700* (8) and *hisP9016* (26), which have been isolated as suppressors of *hisJ5625*, a mutation inactivating the interaction domain of the histidine-binding protein (8) (Fig. 2). A third *hisJ5625* suppressor, *hisP9083*, lay further away and is not shown in Fig. 2. Plasmid mutation *hisP9087* changes the same codon as suppressor mutation *hisP5700*, and *hisP9086* is only one codon away from *hisP9016*, another suppressor mutation.

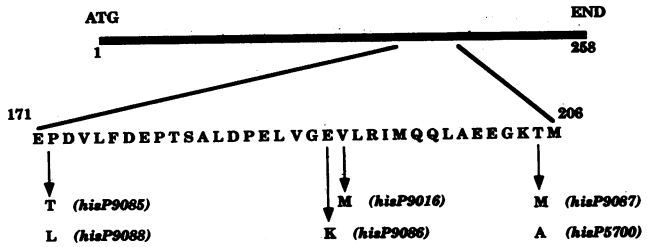


FIG. 2. Locations of *hisP* mutations. The locations and nature of the amino acid changes resulting from the mutations in GA22, GA23, GA24, and GA25 (*hisP9085*, *hisP9086*, *hisP9087*, and *hisP9088*, respectively) and of mutations *hisP5700* and *hisP9088* are shown. Amino acid residues are denoted with the single-letter code. The line represents the HisP amino acid sequence. The region of interest is shown in detail.

**Comparison with *hisP* suppressor mutations.** The closeness between the locations of the plasmid mutations and the known suppressors of the *hisJ5625* interaction mutation suggested that these two classes of mutations are related. In particular, the possibility arose that the mutations obtained from the different selection procedures have the common property of allowing histidine transport in the absence of binding proteins if the membrane components are present at high levels, but of requiring a periplasmic binding protein if the membrane components are present at low levels. This hypothesis was tested after transferring the chromosomal suppressor mutations to the plasmid and vice versa, transferring the plasmid mutations onto the chromosome in the presence and absence of an intact *hisJ* gene. Suppressor mutations *hisP9016* and *hisP9083* were transferred onto plasmid pFA53 as described in Materials and Methods, resulting in plasmids pFA123 and pFA124, respectively. These were introduced into TA3162, creating strains GA143 and GA144, respectively, which were then tested for D-histidine growth by radial streak at 30 and 37°C. Both strains were able to grow on D-histidine at 37°C as effectively as GA22 (*hisP9085*) (data not shown).

To determine whether the plasmid mutations allow D-histidine growth at chromosomal levels of expression in the absence of HisJ, the mutant and wild-type plasmids were introduced into TA2918, which carries the deletion *ΔhisJ6776*, to yield GA112 through GA115 and TA4181, respectively. In these strains, the plasmid-carried genes were placed by homologous recombination under the control of the chromosomal promoter, thereby being expressed at 30°C. If chromosomal levels of the mutant HisP are sufficient to allow transport in the absence of HisJ, the recombinants should be able to grow on D-histidine at 30°C. However, even after lengthy incubation (5 days) on 2 mM D-histidine at 30°C, none of the recombinant strains containing the plasmids gave rise to D-histidine growers (data not shown), while a control strain, TA271, gave D-histidine-utilizing colonies within 2 days. Thus, recombination of the mutation into the chromosome and expression of the mutant proteins at chromosomal *dhuA1* levels is insufficient to allow growth on D-histidine in the absence of HisJ. This result is consistent with the very poor growth of GA22 through GA25 at 33°C as described above, at which temperature expression from the plasmids is low and comparable to chromosomal levels. Therefore, the plasmid mutations behave like the suppressor mutation *hisP5700* in being unable to transport in the absence of HisJ when expressed at chromosomal levels (8). Thus, despite having been obtained by entirely different selection procedures, the plasmid mutations and the chromosomal suppressor mutations seem to form a single class with respect to their location and to their ability to transport

TABLE 5. Growth rate of plasmid mutants in the presence and absence of HisJ

| Strain | Relevant genotype | Plasmid | HisJ[a] | Doubling time[b] (min) |
|---|---|---|---|---|
| GA96 | Δ*his*(*QMP*)*5575* | pFA53 (wild type) | + | 64 |
| GA97 | Δ*his*(*QMP*)*5575* | pFA108 (*hisP9085*) | + | 61 |
| GA98 | Δ*his*(*QMP*)*5575* | pFA109 (*hisP9086*) | + | 60 |
| GA99 | Δ*his*(*QMP*)*5575* | pFA110 (*hisP9087*) | + | 62 |
| GA100 | Δ*his*(*QMP*)*5575* | pFA111 (*hisP9088*) | + | 140 |
| GA21 | Δ*his*(*JQMP argT ubiX*)*6631* | pFA53 (wild type) | − | NG |
| GA22 | Δ*his*(*JQMP argT ubiX*)*6631* | pFA108 (*hisP9085*) | − | >300 |
| GA23 | Δ*his*(*JQMP argT ubiX*)*6631* | pFA109 (*hisP9086*) | − | 630 |
| GA24 | Δ*his*(*JQMP argT ubiX*)*6631* | pFA110 (*hisP9087*) | − | 430 |
| GA25 | Δ*his*(*JQMP argT ubiX*)*6631* | pFA111 (*hisP9088*) | − | 270 |
| GA80 | | pFA53 (wild type) | HisJ5625 | 83 |
| GA81 | | pFA108 (*hisP9085*) | HisJ5625 | 64 |
| GA82 | | pFA109 (*hisP9086*) | HisJ5625 | 72 |
| GA83 | | pFA110 (*hisP9087*) | HisJ5625 | 70 |
| GA84 | | pFA111 (*hisP9088*) | HisJ5625 | 76 |

[a] Symbols: +, wild-type HisJ; −, no HisJ.
[b] Growth was measured on 300 μM D-histidine at 37°C. GA21 to GA25 and GA96 to GA100 were assayed in one experiment; GA80 to GA84 were assayed in another. NG, No growth.

D-histidine in the absence of HisJ and LAO when the respective membrane proteins are overexpressed.

**Interaction with wild-type HisJ.** A characteristic of the chromosomal suppressor mutations is their ability to utilize HisJ to allow growth on D-histidine when expressed at chromosomal levels; in fact, they are entirely dependent on the presence of HisJ (8). This property indicates that the mutated membrane complex, even if expressed at chromosomal levels, is still able to interact and function with the binding protein, allowing transport. In order to determine whether the plasmid mutations behave similarly in this property also, each mutant plasmid was introduced into TA1708, a strain containing a chromosomal deletion (Δ*his5549*) that eliminates part of *hisM* and all of *hisP* but has an intact *hisJ* gene. These derivatives (GA101 through GA105) did not grow on D-histidine at 30°C because there is no expression from the plasmid. However, by recombination into the chromosome, the mutant gene is placed under the control of the chromosomal promoter; this would give rise to colonies that can grow on D-histidine at 30°C if the mutant HisP can function with HisJ. That this recombination test works properly was shown by obtaining wild-type recombinants from all of these mutants with deletions that do not cover the mutations (data not shown). Two of the mutations, *hisP9085* and *hisP9086*, generated D-histidine-utilizing recombinants and therefore could function when expressed at chromosomal levels in the presence of HisJ, thus behaving like the previously isolated chromosomal suppressor *hisP5700*. Chromosomal expression of *hisP9087* and *hisP9088* was insufficient to allow growth even in the presence of HisJ. It should be noted that since neither mutation gave growth in the absence of HisJ, the lack of growth in its presence does not indicate an inhibitory action of HisJ.

The activity of the plasmid mutants in the presence of HisJ under conditions of overexpression of the membrane proteins was also tested. If the mutant membrane complexes can still interact with HisJ, growth of the plasmid mutants on D-histidine under conditions of overexpression should be better in the presence of HisJ than in its absence. The mutant plasmids and pFA53 were transduced into TA1741, which expresses HisJ but carries a deletion covering *hisQ*, *hisM*, and *hisP*. The growth of the resulting strains, GA96 to GA100, on 300 μM D-histidine at 37°C is shown in Table 5. All four mutant strains showed better growth in the presence of HisJ than in its absence; three of them had rates similar to those afforded by the wild type (GA96) when overexpressed and in the presence of HisJ. Since *hisP9087* and *hisP9088* do not allow growth in the presence of HisJ when they are expressed at chromosomal levels, HisP must be the limiting component under these conditions. The *hisP* suppressor mutations *hisP9016* and *hisP9083* were also tested under overexpressing conditions in the presence of HisJ (in strains GA145 and GA146) and gave doubling rates of about 60 min, i.e., comparable to those of GA96 through GA99 (data not shown).

In conclusion, all these mutations, despite their different origins, belong to one general class in which the mutant HisP protein, if produced in large amounts, can function in the absence of HisJ. All of these HisP proteins function in the presence of HisJ, some even when produced in chromosomal amounts.

**Interaction with mutant HisJ proteins.** Even though these HisP mutant proteins allow transport in the absence of HisJ, their performance can be improved by the presence of HisJ. In some cases (*hisP9087* and *hisP9088*) the improvement was dependent on the elevation of the levels of the membrane-bound proteins, suggesting that the latter have a lesser ability to interact with HisJ. Therefore, it was of interest to determine the ability of the plasmid mutants to act as suppressors of *hisJ5625* (8). The plasmids were introduced into TA308, which carries the *hisJ5625* mutation (and a normal complement of wild-type membrane proteins). While TA308 is unable to grow on D-histidine (16), the resulting strains, GA80 through GA84, could grow on it (Table 5). The combination of HisJ5625 with overexpressed wild-type membrane proteins from pFA53 (in GA80) allowed an intermediate level of growth. This result is consistent with the interpretation that the defect in HisJ5625 is in the affinity of the liganded binding protein for the membrane complex. GA81 through GA84 grew much better than GA22 through GA25 (no HisJ). This included even the *hisP9088* mutant, which was not as effective as the other plasmid mutants in using wild-type HisJ. Thus, HisJ5625 preferred mutant to wild-type HisP in all cases, and when combined with *hisP9088* it functioned better than wild-type HisJ. These data indicate that all of these *hisP* mutations are located in regions of HisP that are affected by interactions with the binding protein.

**Interaction with LAO.** The *hisP* mutations might exhibit a

TABLE 6. Growth on D-histidine in the presence of LAO

| Strain[a] | Plasmid | Growth[b] (mm) |
|---|---|---|
| GA152 | pFA53 (wild type) | 19 |
| GA153 | pFA108 (*hisP9085*) | 19 |
| GA154 | pFA109 (*hisP9086*) | 19 |
| GA155 | pFA110 (*hisP9087*) | 11 |
| GA156 | pFA111 (*hisP9088*) | 4 |
| GA157 | pFA123 (*hisP9016*) | 19 |
| GA158 | pFA124 (*hisP9083*) | 19 |
| GA21 | pFA53 (wild type, no LAO) | 0 |
| GA25 | pFA111 (*hisP9088*, no LAO) | 4 |

[a] All strains carry the *ntrB139* mutation, which elevates the expression of LAO, except for GA21 and GA25, which are deleted for the *argT* gene, which codes for LAO.

[b] Cells were radially streaked from a disk containing 1.0 μmol of D-histidine and incubated at 37°C for 24 h.

similar spectrum of effects when combined with LAO, a protein that transports lysine, arginine, and ornithine through the Q/M/P complex, and therefore must share many of the interaction determinants with HisJ. Since LAO functions in the transport of D-histidine, we measured the ability of each of the overproduced mutant HisP proteins to utilize LAO to allow growth on D-histidine by introducing each plasmid into GA151, which has constitutive levels of LAO and no HisJ, yielding GA152 through GA158. Table 6 shows that wild-type HisP allowed strong D-histidine growth when combined with LAO, as expected. HisP9016, HisP9083, HisP9085, and HisP9086 all functioned well with LAO. However, HisP9087 and HisP9088 gave poorer growth; HisP9088 gave growth after 24 h which was no better than in the absence of LAO. Thus, the *hisP* mutations show the same overall pattern of interaction with LAO as they do with wild-type HisJ.

## DISCUSSION

One of the unanswered questions concerning the mechanism of action of periplasmic permeases is whether the membrane-bound components allow translocation of the substrate by forming a channel with no or relatively little substrate specificity (pore model) or by way of specific membrane-binding sites (binding-site model). The former model implies that the specificity of these permeases is entirely due to the periplasmic protein, while the binding-site model imparts some specificity function also to one or more of the membrane components. In support of the binding-site model are mutations in HisQ and HisM (the hydrophobic membrane-bound components of the histidine permease) that have resulted in a change in the specificity of transport (12). Even more compelling are mutations altering the maltose permease hydrophobic components (MalF and MalG) that allow transport in the absence of the maltose-binding protein, suggesting that these proteins carry substrate-binding sites (26). In this study we characterized several histidine permease mutants able to transport substrate in the absence of the periplasmic components. The mutants were derived from a strain that produces high levels of the membrane components, and the mutations were located in *hisP*. These mutants transport D-histidine in the absence of binding proteins with relatively low rates and affinity. The spectrum of specificity of these mutants is narrower than that of the wild-type permease, since it excludes arginine, lysine, and *N*-acetyl-L-histidine from the substrates transported. The apparent affinity of the mutant transport complexes is greatly improved when they are placed in the presence of binding proteins. These findings are consistent with but do not prove the presence of a binding site(s) on the membrane complex that can be mutated to receive the substrate directly, thus lending support to the binding-site model. There has been no previous evidence for a role for the hydrophilic membrane component in determining substrate specificity. We tentatively suggest that HisP contributes to the formation of a substrate-binding site, either directly or indirectly.

The finding of a close relationship, with respect to both properties and DNA locations, between these mutants and previously isolated suppressors of binding protein interaction mutants is interesting. Our previous identification of this class of suppressor mutations had led us to conclude that the binding protein HisJ makes direct contact with HisP, the suppressor HisP protein resulting in a better fit with an altered interaction site on HisJ (8). It now appears that these suppressor proteins, when present in high amounts, also allow transport in the total absence of HisJ. This finding could be explained if the mutant HisP allowed both a better interaction with an altered HisJ and a modulation of the protein domain(s) that forms a histidine-binding site within the membrane. The substrate-binding site(s) may be restricted entirely to either HisP or HisQ/HisM, or it may be shared by more than one of these proteins. If it were entirely within HisQ/HisM, then it would be necessary to postulate that HisP exerts its action indirectly. We are in the process of characterizing several HisJ mutant proteins that cannot interact with the membrane complex, using a cross-linking assay (24); one of these mutant proteins is not suppressed by *hisP5700*, indicating an allele-specific HisP suppression of HisJ interaction defects (22a). The data presented in Tables 5 and 6 also suggest some allele specificity. However, this may not be a sufficient argument to conclude unequivocally that HisP and HisJ interact directly.

An important point to be addressed is whether the HisP protein, which binds ATP and therefore must face the cytoplasm, has any direct involvement in events at the periplasmic face of the membrane. We have obtained preliminary evidence that HisP is accessible to proteolytic digestion at the periplasmic surface (15b). In addition, we have developed a structural model of HisP which includes a moderately hydrophobic domain that is likely to traverse the membrane embedded between HisQ and HisM (4a, 21). A domain of HisP might therefore be exposed at the periplasmic surface and be involved in binding the substrate and/or interacting with HisJ. The region of HisP where the mutations described here are located might define or be close to the domain that is accessible to the periplasm. This region is not large: the mutations in the four plasmid mutants and two of the suppressor mutants fell within a 34-residue sequence in the C-terminal one-third of HisP, and within this region they were clustered into three spots.

It is premature to hypothesize how a mutant protein can have an effect on both a binding and an interaction site. Perhaps a substrate-binding site on wild-type HisP cannot receive the substrate efficiently when it is offered by a liganded HisJ that is defective in interaction (which, for example, could keep the substrate too far from HisP, or possibly obliterate its route of access). A mutation in the HisP substrate-binding site might make this site more accessible to the periplasm, thus improving the interaction with either free substrate or liganded mutant HisJ. This hypothetical site in the wild-type state might routinely receive and translocate substrate coming from the interacting liganded binding protein, but it could also accept free substrate

directly if interaction of the complex with unliganded binding protein "opened" a membrane-bound site.

An alternative explanation for the properties of these mutants might be that the membrane complex has acquired the ability to undergo spontaneously the conformational changes that lead to active transport. In this respect, it is interesting that several of the mutations are located close to portions of HisP that are predicted to be part of the nucleotide-binding site (21). Thus, it is possible that the mutations result in an ability to hydrolyze ATP independently from the stimulus of the liganded binding protein; this might result in a concomitant increased accessibility of a pore or of a binding site.

Understanding the molecular basis of the defect in the mutants we have described clearly requires additional experimentation. Among the experimental approaches to prove the existence of a membrane-bound substrate-binding site, whether on HisQ, HisM, or HisP, are the demonstration of a substrate-binding activity by the purified membrane-bound components and of transport activity in reconstituted proteoliposomes (9) by using purified mutant membrane complex in the absence of added periplasmic components. In such a case, it must still be postulated that the membrane-bound complex has a domain reserved for specific substrate recognition.

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