JOURNAL OF BACTERIOLOGY, Apr. 1979, p. 218-234
0021-9193/79/04-0218/17$02.00/0

Vol. 138, No. 1

Nitrogen Control in *Salmonella typhimurium*: Co-Regulation of Synthesis of Glutamine Synthetase and Amino Acid Transport Systems

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Received for publication 1 January 1979

Nitrogen control in *Salmonella typhimurium* is not limited to glutamine synthetase but affects, in addition, transport systems for histidine, glutamine, lysine-arginine-ornithine, and glutamate-aspartate. Synthesis of both glutamine synthetase and transport proteins is elevated by limitation of nitrogen in the growth medium or as a result of nitrogen (N)-regulatory mutations. Increases in the amounts of these proteins were demonstrated by direct measurements of their activities, by immunological techniques, and by visual inspection of cell fractions after gel electrophoresis. The N-regulatory mutations are closely linked on the chromosome to the structural gene for glutamine synthetase, *glnA*; we discuss the possibility that they lie in a regulatory gene, *glnR*, which is distinct from *glnA*. Increases in amino acid transport in N-regulatory mutant strains were indicated by increased activity in direct transport assays, improved growth on substrates of the transport systems, and increased sensitivity to inhibitory analogs that are transported by these systems. Mutations to loss of function of individual transport components (*hisJ*, *hisP*, *glnH*, *argT*) were introduced into N-regulatory mutant strains to determine the roles of these components in the phenotype and transport behavior of the strains. The structural gene for the periplasmic glutamine-binding protein, *glnH*, was identified, as was a gene *argT* that probably encodes the structure of the lysine-arginine-ornithine-binding protein. Genes encoding the structures of the histidine- and glutamine-binding proteins are not linked to *glnA* or to each other by P22-mediated transduction; thus, nitrogen control is exerted on several unlinked genes.

Many bacteria, including those of the enteric group, elevate synthesis of glutamine synthetase in response to limitation of nitrogen in the growth medium (16, 22, 23, 48, 80); this elevation presumably reflects the role of glutamine synthetase together with glutamate synthase (GOGAT) in the assimilation of NH_4^+ into glutamate under nitrogen-limiting conditions (22, 23, 49, 50). Some enteric bacteria, notably *Klebsiella aerogenes* and *Klebsiella pneumoniae*, show similar "nitrogen control" of the synthesis of other enzymes that lead to formation of glutamate, e.g., the histidine (*hut*) and proline (*put*) degradative enzymes, and nitrogenase (50, 56; reviewed in references 46 and 47). *Salmonella typhimurium* is not known to show nitrogen control of the synthesis of enzymes other than glutamine synthetase (24, 28, 51, 56). However, this paper presents evidence that *S. typhimurium* indeed does show nitrogen control of the synthesis of four amino acid transport systems with periplasmic binding protein components; these systems function in the transport of histidine, glutamine, lysine-arginine-ornithine, and glutamate-aspartate, respectively. To quantitate accurately the increase in synthesis of the transport systems, we have measured the elevation of their periplasmic binding protein components in response to nitrogen limitation.

Nitrogen (N)-regulatory mutant strains of *S. typhimurium* elevate expression of the four transport systems and of glutamine synthetase even under conditions of nitrogen excess. Mutant strains of *K. aerogenes* with similar properties—elevated expression of glutamine synthetase and many other enzymes subject to nitrogen control under all growth conditions (GlnC phenotype [23, 55])—were reported to have mutations within *glnA*, the structural gene for glutamine synthetase (16, 17, 70). The mutations we have studied in *Salmonella* are closely linked to *glnA* by P22-mediated transduction; however,

we favor the alternative hypothesis that they lie in the gene for a positive regulatory factor, *glnR*, which is distinct from *glnA* (S. Kustu, E. Garcia, S. Bancroft, N. McFarland, S. Hui, and D. Burton, manuscript in preparation).

Relatively little is known about regulation of amino acid transport systems in the enteric bacteria, particularly with regard to general controls. Synthesis of several amino acid transport systems is specifically regulated by availability of substrate in the growth medium (27, 58, 60). In many cases the mechanism for specific control is at least partly separable from that controlling the biosynthetic pathway for the amino acid (26, 58, 59, 60). In the case of branched-chain amino acid transport, $tRNA^{leu}$ has an important role in specific regulation (57, 58). Properties of N-regulatory mutant strains described in this paper indicate that synthesis of histidine, glutamine, arginine, and glutamate transport systems is subject to general control by availability of nitrogen in the growth medium.

MATERIALS AND METHODS

Chemicals. L-[*U*-^{14}C]glutamine (251 mCi/mmol), L-[3,4-^{3}H]glutamine (32.5 Ci/mmol), L-[2,3-^{3}H]arginine (21.5 Ci/mmol), L-[3-^{3}H]histidine (10.9 Ci/mmol), L-[3,4-^{3}H]glutamic acid (45.0 Ci/mmol), and L-[3,4-^{3}H]leucine (60 Ci/mmol) were obtained from New England Nuclear Corp. D-2-Hydrazino-3-(4-imidazolyl) propionic acid (HIPA) was synthesized from L-histidine (68). Azaserine (*O*-diazoacetyl-L-serine) was obtained from the National Cancer Institute (Cancer Chemotherapy National Service Center, Bethesda, Md.) or was purchased from P-L Biochemicals. D,L-α-Methylglutamate was purchased from Mann Research Labs. D-Glutamate, D-histidine, and L-glutamic acid γ-hydrazide (γ-glutamylhydrazide) were purchased from Sigma. L-Arginyl-L-arginine triacetate and *N*-formyl-L-histidine were purchased from Vega-Fox Biochemicals, Tucson, Ariz. Diethylaminoethyl (DEAE)-cellulose DE52 was purchased from Whatman. Rabbit muscle phosphorylase b was a kind gift from K. Stam and E. Krebs. Antisera to the *Escherichia coli* glutamine-binding protein and the *E. coli* glutamate-binding protein were kindly provided by C. E. Furlong. Immunoplates, pattern C, for Ouchterlony double diffusion were obtained from Hyland Division of Travenol Laboratories, Costa Mesa, Calif. Carboxymethyl (CM) cellulose CM52 and DEAE-cellulose DE52 were obtained from Whatman.

Media and growth of strains. Minimal medium refers to the salts mixture described previously (13) with 0.4% glucose added as carbon source; for a condition of nitrogen excess 10 mM NH_4Cl and 3 mM glutamine were added as nitrogen sources, and for a condition of nitrogen limitation 10 mM glutamate was added as the nitrogen source (25). The doubling time of *S. typhimurium* on glutamate as nitrogen source is approximately 4 h. Medium E of Vogel and Bonner (74), which contains 17 mM NH_4^+ as a nitrogen source, was used in some experiments; 0.4% glucose was added as a carbon source. For determination of growth rates, inoculum cultures were grown overnight in medium E plus 0.4% glucose and then diluted at least 50-fold into minimal medium containing the supplements specified. Cultures were incubated at 37°C with aeration. Bacterial growth was monitored turbidimetrically by measuring optical density at 650 nm. Growth tests on petri plates were done by placing a filter paper disk containing the compound to be tested (either as a growth substrate or inhibitor) on a plate seeded with 10^8 bacteria (41, 64). The diameter of the zone of growth or inhibition was measured in centimeters after 18 to 24 h of incubation at 37°C.

Isolation and genetic analysis of N-regulatory mutant strains. The strains constructed for this work (Table 1) were derived from *S. typhimurium* strain LT2. Strains with N-regulatory mutations near *glnA*, the structural gene for glutamine synthetase (43), were isolated in three ways. Selection 1 was for increased glutamine synthetase activity. Clones resistant to glutamate inhibition of two *glnD* strains, SK103 and SK111, were isolated as described previously (13). Strains with *glnD* mutations lack the activity of P_{IIA} uridylyltransferase (13), one of the enzymes required for reversible covalent modification of glutamine synthetase. These strains accumulate the adenylylated (less active) form of glutamine synthetase; in addition, they have less glutamine synthetase than does wild type. They are glutamine bradytrophs and are sensitive to growth inhibition by 10 mM glutamate (reason unknown) (13); about half the spontaneous glutamate-resistant suppressors derived from the *glnD* strains (33 out of 54), including those that were studied for this work, had more glutamine synthetase than did wild type. The strain that had the highest glutamine synthetase activity, SK210 (*gln-128 glnD79 hisF645*), and strains derived from it were chosen for detailed studies.

Selection 2 was for increased histidine transport. Fast-growing papillae were isolated from strain TA2944 [Δ(*hisJ-dhuA*)*5643 hisF645*] around a disk containing 1 μmol of *N*-formyl histidine as the histidine source. Transport, and therefore utilization, of *N*-formyl histidine is limited in TA2944 by decreased synthesis of the *hisP* gene product, a component of the high-affinity histidine transport system. Clones that grew more rapidly on *N*-formyl histidine were purified and screened for increased sensitivity to the histidine analog HIPA (7, 12) and for fast growth on arginine as nitrogen source (41), both of which are indicative of increased function of the high-affinity histidine transport system.

Selection 3 was for increasd arginine transport. Fast-growing papillae were selected in strains TA1834 [Δ(*hisJ-dhuA*)*5650 hisF645*] and TA1811 [Δ(*hisJ-dhuA*)*5650*] on minimal plates containing 3 mM arginine as nitrogen source (and 1% glucose as carbon source). Transport of arginine is limited in the parent strains by decreased synthesis of the *hisP* gene product (41).

N-regulatory mutations in strains isolated by all three selection procedures were transferred by transduction to strain SK35 [Δ(*glnA-glnR*)*60 hisF645*] by selecting for glutamine prototrophy and screening transductant clones for elevated levels of glutamine

TABLE 1. *Bacterial strains*

Strain	Genotype	Parents	Source
TA1772	Wild type		G. F.-L. Ames
TA831	Δ*hisF645*		G. F.-L. Ames
SK35	Δ(*glnA-glnR*) *60*[a] *hisF645*		(25)
SK103	*glnD79 hisF645*		(13)
SK111	*glnD96 hisF645*		(13)
SK207	*gln-125 glnD96 hisF645*	SK111	Spontaneous
SK208	*gln-126 glnD96 hisF645*	SK111	Spontaneous
SK209	*gln-127 glnD96 hisF645*	SK111	Spontaneous
SK210	*gln-128 glnD79 hisF645*	SK103	Spontaneous
SK211	*gln-125 hisF645*	SK35, SK207	Transduction
SK212	*gln-126 hisF645*	SK35, SK208	Transduction
SK213	*gln-127 hisF645*	SK35, SK209	Transduction
SK214	*gln-128 hisF645*	SK35, SK210	Transduction
TA2944	Δ(*hisJ-dhuA*)*5643*[b] *hisF645*		(11)
TA3173	*gln-139 his-5643 hisF645*	TA2944	Spontaneous
SK295	*gln-139 hisF645*	SK35, TA3173	Transduction
TA3290	*gln-139*	TA3239, LT2	Transduction
TA3218	*gln-139 dhuA1 hisF645*	TA3173, TA271	Transduction
TA3292	*gln-139 dhuA1*	TA3218, LT2	Transduction
TA1834	Δ(*hisJ-dhuA*)*5650*[b] *hisF645*	TA1637, TA1811	Transduction
TA1811	*his-5650*	TA1014	(11)
TA3343	*gln-481 his-5650 hisF645*	TA1834	Spontaneous
TA3344	*gln-482 his-5650 hisF645*	TA1834	Spontaneous
TA3345	*gln-483 his-5650 hisF645*	TA1834	Spontaneous
TA3219	*argT526 gln-139 his-5643 hisF645*	TA3173	Spontaneous
TA3175	*gln-139 zei-102*::Tn*10*[c] *dhuA1* Δ*hisP6712 hisF645*	TA3173, TA3166	Transduction
TA3286	*gln-139 argT526 dhuA1 hisF645*	TA3219, TA3175	Transduction
TA3293	*gln-139 argT526 dhuA1*	TA3286, LT2	Transduction
TA3296	*gln-139 dhuA1 hisJ5601 hisF645*		
TA3287	*gln-139 argT526 dhuA1 hisF645 zei-102*::Tn*10*		
TA3297	*gln-139 argT526 dhuA1 hisJ5601 zei-102*::Tn*10 hisF645*	TA3296, TA3287	
TA3298	*gln-139 argT526 dhuA1 hisJ5601 zei-102*::Tn*10*	TA3297, LT2	
TA1650	*dhuA1*[d] *his J5601*		
SK269	*dhuA1 hisJ5601 hisF645 zei-102*::Tn*10*	TA1646, TA3088	Transduction
SK270	*gln-128 dhuA1 hisJ5601 hisF645 zei-102*::Tn*10*	SK214, SK269	Transduction
TA3174	*gln-139 dhuA1 hisJ5601 hisF645 zei-102*::Tn*10*	TA3173, SK269	Transduction
TA3285	*gln-139 dhuA1 hisJ5601 zei-102*::Tn*10*	TA3284, LT2	Transduction
SK427	*dhuA1* Δ*hisP6712*		
TA3294	*gln-139 dhuA1* Δ*hisP6712 zei-102*::Tn*10*	TA3175, LT2	
TA3067	*dhuA1 hisP6757*::Tn*10 his F645*		
TA3090	*hisP6757*::Tn*10 hisF645*		F. Ardeshir
SK425	*gln-128 dhuA1 hisP6757*::Tn*10*	SK214, TA3067	
SK426	*gln-128 hisP6757*::Tn*10*	SK214, TA3090	
SK359	*glnH141 gln-128 hisF645*	SK214	Spontaneous
SK364	*glnH146 gln-128 hisF645*	SK214	Spontaneous
SK367	*glnH149 gln-128 hisF645*	SK214	Spontaneous
SK369	*glnH151 gln-128 hisF645*	SK214	Spontaneous
SK273	*zig-205*::Tn*10 hisF645*	SK35, pooled tetracycline-resistant clones[f]	Transduction
SK383	*glnH141 hisF645 zig-205*::Tn*10*	SK359, SK273	Transduction
SK384	*glnH146 hisF645 zig-205*::Tn*10*	SK364, SK273	Transduction

[a] See Materials and Methods.

[b] The Δ(*hisJ-dhuA*)*5643* and Δ(*hisJ-dhuA*)*5650* mutations cover the operator region (*dhuA*) for the histidine transport operon as well as a portion of the gene (*hisJ*) for the periplasmic histidine-binding protein J (11).

[c] *zei-102*::Tn*10* is 80% linked to the histidine transport operon by P22-mediated transduction and is located clockwise of *dhuA*.

[d] The *dhuA1* mutation is in the promoter for the histidine transport operon (11) and elevates expression of the operon (7, 40); *hisJ* mutations have been isolated only in strains that carry the *dhuA1* mutation and have not been separated from it.

[e] *zig-205*::Tn*10* is 40% linked to *glnA* by P22-mediated transduction.

[f] Phage lysate on ~2,000 tetracycline-resistant clones formed by translocation of the Tn*10* element into the *Salmonella* chromosome (39).

synthetase or ability to grow on D-histidine as the histidine source (see Results). Linkage of N-regulatory mutations to Δ(*glnA-glnR*)*60* was determined by scoring transductant clones for growth on D-histidine. The Δ(*glnA-glnR*)*60* mutation, which is known to cover a portion of *glnA* (33), also fails to recombine with two point mutations in *glnR* (*glnR174* and *glnR183*) (S. Hui and S. Kustu, unpublished data). Regulatory mutations are designated *gln*− since they are probably not within *glnA*, the structural gene for glutamine synthetase, but may be in the closely linked regulatory gene *glnR*. (The regulatory mutations do not cause a glutamine requirement.) Phage was P22 HT *int201* (67).

Isolation of a strain with an *argT* mutation. Strain TA3219 (*argT526 gln-139 his-5643 hisF645*), which had lost the periplasmic lysine-arginine-ornithine (LAO)-binding protein (so-identified as discussed in Results), was isolated from N-regulatory mutant strain TA3173 (*gln-139 his-5643 hisF645*) by selecting for HIPA resistance, a selection for decreased function of the high-affinity histidine transport system. Strain TA3173 was seeded on minimal plates supplemented with carnosine (~0.03 mM), and disks containing HIPA (0.5 and 1 μg) were placed on the plates. Papillae growing in the zones of inhibition were purified, tested for the presence of the LAO protein by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and analyzed genetically. Several strains which had lost the LAO elevation characteristic of N-regulatory mutant strains (see Fig. 4) had mutations in the *glnA* or *glnF* (33) regions (S. Hui and S. Kustu, unpublished data) and were not studied further in this context. Other strains had mutations linked to the histidine transport operon (where all prior HIPA-resistant mutations were located [11, 12] but outside the operon). One such strain, TA3219, was studied in more detail. The mutation in this strain (*argT526*) is 98% linked to *dhuA*, the promoter for the histidine transport operon, and was found by three-point recombination tests to be located clockwise from *dhuA* (G. F.-L. Ames, unpublished data). We have assumed that the *argT* gene is the structural gene for the LAO protein, although proof awaits the identification of an *argT* strain with an altered LAO protein. The mutation in the histidine transport operon, *his-5643*, was removed from TA3219 by transducing it to growth on D-histidine as the histidine source with phage grown on TA3175 (*gln-139 zei-102*::Tn*10 dhuA1 hisP6712 hisF645*) and choosing a tetracycline-sensitive transductant clone that lacked the LAO band on SDS-PAGE analysis (TA3286).

Isolation of an *argT hisJ* strain. Strain TA3297 (*argT526 hisJ5601 dhuA1 gln-139 hisF645 zei-102*::Tn*10*) was isolated from strain TA3296 (*hisJ5601 dhuA1 gln-139 hisF645*) by transducing it to tetracycline resistance with phage prepared on strain TA3287 (*argT526 dhuA1 gln-139 hisF645 zei-102*::Tn*10*) and screening recombinant clones for failure to grow on D-histidine. These were further analyzed by gel electrophoresis for lack of both the LAO and J proteins.

Isolation and genetic analysis of strains with *glnH* mutations. Strains (*glnH*) that had lost the activity of the periplasmic glutamine-binding protein were isolated among derivatives of N-regulatory mutant strain SK214 that had acquired resistance to the glutamine analog γ-glutamylhydrazide (2 μmol on a filter paper disk [75]). A total of 200 spontaneous resistant clones (from 20 independent cultures of strain SK214) were tested for their ability to grow on D-histidine as the histidine source and on arginine as the nitrogen source. Four clones that had lost these abilities had mutations in the *glnA* or *glnF* (33) regions (S. Hui and S. Kustu, unpublished data) and were not studied further in this context. The remaining γ-glutamylhydrazide-resistant clones were screened for glutamine-binding protein antigen and glutamine-binding activity. Samples were prepared as follows. Cells were harvested by centrifugation from 2-ml cultures grown in medium E + 0.4% glucose, cell pellets were suspended in the small volume of medium that could not be drained from the tube, and the cells were treated with 20 μl of chloroform-toluene (1:1 by volume) (35) for at least 15 min at room temperature. An 8-μl portion of each cell suspension was then screened for the presence of glutamine-binding protein antigen by Ouchterlony double diffusion by using 5 μl of antiserum raised against the *E. coli* glutamine-binding protein. About 10% of the clones checked (17 out of 180) failed to form a precipitin band with this antiserum. A total of 120 clones were also screened for glutamine-binding activity by equilibrium dialysis. To prepare samples, 0.5 ml of 10 mM Tris-chloride buffer (pH 7.3) was added to the remainder of each chloroform-toluene–treated cell suspension, the suspension was heated at 60°C for 20 min and allowed to cool for several hours, and cells were removed by centrifugation. (It is likely that neither heating nor removal of the cells was necessary.) Strains that lacked glutamine-binding protein antigen and one additional strain that had the antigen SK359 had <3% as much binding activity as strain SK214. Mutations in these strains are designated *glnH*. All other strains had >50% as much glutamine-binding activity as strain SK214.

Linkage of *glnH* to *glnA* was determined by transducing strain SK35 [Δ(*glnA-glnR*)*60 hisF645*] to glutamine prototrophy with phage grown on strain SK359 or strain SK364 and scoring transductant clones for γ-glutamylhydrazide resistance (by radial streak [5] against 0.5 μmol of analog in a disk). Since all transductant clones inherited the N-regulatory mutation *gln-128*, which is 100% linked to Δ(*glnA-glnR*)*60* (Results; S. Kustu, unpublished data) and which confers γ-glutamylhydrazide sensitivity, the number of γ-glutamylhydrazide–resistant transductants is a measure of the linkage of *glnH* to *gln-128* and therefore to *glnA*. Linkage of *glnH* to *hisP* was determined by transducing strains SK359 and SK364 and their parent strain SK214 to tetracycline resistance with phage grown on a strain which carries a Tn10 insertion in *hisP* (TA3067) and scoring transductant clones for γ-glutamylhydrazide sensitivity. The number of transductant clones (all of which are *hisP*) that become γ-glutamylhydrazide sensitive is a measure of the linkage of *glnH* to *hisP* since strain SK425 (*gln-128 dhuA1 hisP6757*::Tn*10 hisF645*), which was derived from strain SK214, is γ-glutamylhydrazide sensitive.

The *gln-128* mutation was removed from strains SK359 and SK364 by transducing them to tetracycline

resistance with phage grown on a strain with a Tn10 insertion 40% linked to the *glnA* region (SK273) and choosing transductant clones that had lost the ability to grow on D-histidine as the histidine source.

Glutamine synthetase assays. Total activity of glutamine synthetase, independent of degree of adenylylation, was determined in crude extacts (11) by using the γ-glutamyl transfer assay of Stadtman et al. (69) with the modifications described (43). Protein was determined by the method of Lowry et al. (45) after precipitation with 5% trichloroacetic acid.

Assays of transport. The transport of L-[^{3}H]histidine (1 to 5 nM, 4.46×10^8 cpm/μmol) by the high-affinity histidine transport system was assayed at 37°C by the "growing cells" method in which incorporation of low concentrations of exogenous amino acid into the protein of exponentially growing cells is measured (3, 5). Rates were linear with time for at least 1 min and with cell concentration. (Cell concentrations between 5×10^{-6} and 20×10^{-6} g [dry weight]/ml were used.) Rates are expressed in micromoles per minute per gram (dry weight) of cells. The conversion factor for calculating dry weight was 4.7×10^{-4} g of dry weight/ml at an optical density at 650 nm of 1.

The transport of L-[^{14}C]glutamine (8 μM, 8.3×10^7 cpm/μmol) was assayed at 23°C by the method of Weiner and Heppel (75). Rates were linear with time and with cell concentration. (Cell concentrations between 5×10^{-5} and 50×10^{-5} g [dry weight] of cells per ml of reaction mixture were used depending on the strain.) Rates are expressed in micromoles per minute per gram (dry weight) of cells.

Preparation of periplasmic fractions and binding assays. Periplasmic fractions were prepared by subjecting stationary cells (30 to 230 ml at optical density at 650 nm of about 2) to osmotic shock by the procedure of Nossal and Heppel (52) with the modifications described (42). The volume of a shock fluid was 1/10 the volume of culture from which it was prepared. In some cases shock fluids were frozen before their binding activity was assayed. Shock fluids were sterilized by addition of chloroform since the glutamine- and glutamate-binding proteins are retained on membrane filters (Millipore Corp.) (C. E. Furlong, personal communication; S. Hui and S. Kustu, unpublished results). Binding of ^{3}H-labeled L-amino acids was assayed by equilibrium dialysis as described previously (44) at a free amino acid concentration of 10 nM (specific activity approximately 2,000 cpm/pmol). (Shock fluids were predialyzed against a large volume of buffer to remove endogenous amino acids before the binding assays were started.) Shock fluids of strain SK214 (0.5 mg of protein/ml) were diluted about 10-fold, 10-fold, and 40-fold for assays of glutamine, histidine, and arginine binding, respectively, and were not diluted for assays of glutamate binding. Activity is expressed as picomoles of amino acid bound per milligram (dry weight) of cells.

Immunology. Ouchterlony double-diffusion plates were prepared with 5 μl of rabbit antiserum in the center well and 5 μl of crude extracts (13), periplasmic fractions, purified binding proteins, or serial twofold dilutions of such fractions in the outer wells and were incubated at 4°C for 24 h. The amounts of antigen in different crude extracts were compared by determining the highest dilution at which each formed a precipitin band. Extracts with a protein concentration of 15 to 20 mg/ml were prepared from concentrated cell suspensions, since periplasmic fractions from the wild-type strain did not contain high enough concentrations of antigen to give precipitin bands with antisera to all binding proteins. The antisera had been raised against (i) purified *Salmonella* glutamine synthetase; (ii) homogeneous *E. coli* glutamine-binding protein; (iii) homogeneous *E. coli* glutamate-aspartate-binding protein; and (iv) homogeneous *Salmonella* histidine-binding protein. We were able to detect the LAO-binding protein immunologically because we found that it cross-reacts with antibodies raised against the *Salmonella* histidine-binding protein (G. F.-L. Ames, unpublished data). Thus, N-regulatory mutant strains (e.g., SK214 and TA3239), which have elevated levels of both the histidine-binding protein and the LAO-binding protein, gave two precipitin bands with antibody to the histidine-binding protein. N-regulatory mutant strains carrying a *hisJ* mutation (e.g., TA3285) and therefore lacking the histidine-binding protein J gave a single precipitin band (due to the LAO protein) and, similarly, N-regulatory mutant strains carrying an *argT* mutation (e.g., TA3293) and lacking the LAO-binding protein gave a single precipitin band (due to the histidine-binding protein). The precipitin band for the LAO-binding protein formed closer to the antibody well than that for the histidine-binding protein.

SDS-PAGE. SDS-PAGE was performed as described previously (6). The acrylamide concentration was 9%. Samples of shock fluids were prepared as described previously (42). In some cases proteins other than those of interest were first removed from shock fluids by adsorption to DEAE-cellulose. A 1-ml amount of DEAE-cellulose suspended in 1 ml of 10 mM Tris-chloride buffer (pH 7.8) was added to 5 ml of shock fluid, which was allowed to stand for 30 min with occasional mixing. The DEAE was then removed by filtration over glass wool.

Purification of binding proteins. Histidine-binding protein J was purified to homogeneity as described previously (K. D. Noel, Ph.D. Thesis, University of California, Berkeley, 1977). An arginine-binding protein, which also has affinity for lysine and ornithine (LAO protein), was partially purified as follows. Cells (935 g) of TA3218 (*gln-139 dhuA1 hisF645*) grown in medium E (74) + glucose and histidine were disrupted in a Manton-Gaulin homogenizer. The crude supernatant fluid was slowly brought to 60% saturation with solid ammonium sulfate (Mann, ultrapure) and precipitated protein was removed by centrifugation. The supernatant fraction was then brought to 100% saturation with ammonium sulfate and the precipitate was collected. The precipitated protein was dissolved in 10 mM potassium phosphate buffer (pH 7.6), and was dialyzed against 5 mM Tris chloride (pH 8.3). The final solution (1,200 mg of protein) was then applied to a DEAE-cellulose column (4.2 by 26 cm). The column was washed with 400 ml of 5 mM Tris-hydrochloride (pH 8.3) and eluted with a linear gradient of NaCl from 0 to 50 mM in the same buffer. A major peak of arginine-binding activity was detected at 1.1 mmho conductivity, which corresponds to 25 mM NaCl, and the fractions with activity were pooled.

The glutamine- and glutamate-binding proteins were purified as follows. Acetate buffer (pH 4.5, final concentration 10 mM) was added to the latter portion of the flow-through and wash from the DEAE-cellulose column described above, which contained both glutamine and some glutamate-binding activity. This fraction (56 mg of protein) was applied to a CM-cellulose column (1 by 36 cm), which was washed with 10 mM acetate (pH 4.5) and eluted with a linear gradient of 0 to 300 mM NaCl in the same buffer. Fractions with glutamine-binding activity, which were eluted at approximately 70 mM NaCl, were pooled, dialyzed against 10 mM acetate (pH 4.5), and further purified on CM-cellulose by using a shallower linear gradient of NaCl (0 to 100 mM). Fractions with glutamate-binding activity, which were eluted from the first CM-cellulose column at approximately 210 mM NaCl, were pooled.

RESULTS

Phenotype of N-regulatory mutant strains. N-regulatory mutant strains with elevated glutamine synthetase activity and elevated transport of histidine, glutamine, arginine, and glutamate were isolated by three different selection procedures, as described in Materials and Methods. Glutamine synthetase, which was known to be elevated by nitrogen limitation in the wild-type strain, is elevated in N-regulatory mutant strains under all growth conditions. N-regulatory mutant strains carrying the *gln-128* or *gln-139* mutations had 15 to 25 times more glutamine synthetase activity and antigen than the wild-type strain under conditions of nitrogen excess (Table 2). (Similar results were obtained for N-regulatory mutant strains carrying the *gln-125–gln-127* mutations.) Expression of glutamine synthetase in N-regulatory mutant strains was elevated even further under nitrogen-limiting conditions; under these conditions the level of glutamine synthetase in some strains was higher than is ever seen in wild type (Table 2).

TABLE 2. *Glutamine synthetase activities*[a] *and antigen levels*[b] *in wild-type and N-regulatory mutant strains*

Strain and relevant genotype	Growth conditions		
	N-excess		N-limiting (derepressing)[e]
	Very repressing[c]	Repressing[d]	
TA831 (wild-type)[f]	0.07[g]	0.21 (1)	1.86 (1:8)
SK214 (*gln-128*)	1.76 (1:8)	1.84 (1:8)	2.64 (1:16)
SK295 (*gln-139*)	1.15	1.20[h]	
TA2944 (wild-type)[i]		0.30[h]	
TA3173 (*gln-139*)		1.14[h]	

[a] Total glutamine synthetase activity independent of the degree of adenylylation was measured by the γ-glutamyl transfer assay of Stadtman et al. (69) with the modifications described previously (43) and is expressed in micromoles per minute per milligram of protein.

[b] Numbers in parentheses indicate the greatest dilution of extract at which a precipitin band formed with antiglutamine synthetase serum on Ouchterlony double diffusion.

[c] Nutrient broth plus 1 mM glutamine. Unlike N-regulatory mutant strains, strains with *glnA* mutations that cause a loss of (or decrease in) catalytic activity of glutamine synthetase have normal levels of glutamine synthetase antigen under these growth conditions; for physiological reasons the *glnA* strains have elevated levels of glutamine synthetase antigen under "repressing" growth conditions (43; E. Garcia, C. E. Neumann, and S. Kustu, unpublished data).

[d] Minimal medium with 10 mM NH_4^+ and 3 mM glutamine as nitrogen sources.

[e] Minimal medium with 10 mM glutamate as nitrogen source (25; Materials and Methods).

[f] Isogenic with strains SK214 and SK295.

[g] No precipitin band was formed by the undiluted extract.

[h] Grown in medium E (17 mM NH_4^+ as nitrogen source) (74).

[i] Isogenic with strain TA3173.

The following growth characteristics of N-regulatory mutant strains (summarized in Table 3) suggest that they have elevated levels of several transport systems.

(i) Their ability to grow on D-histidine, a substrate of the high-affinity histidine transport system (7, 40), as the histidine source and their increased sensitivity to the analogs HIPA and azaserine, which are known to be transported by the high-affinity histidine transport system (7, 8, 12), suggest that histidine transport is elevated. (Transport is rate-limiting for growth on D-histidine [7, 40].)

(ii) Their increased sensitivity to the analog γ-glutamylhydrazide, which is transported by the glutamine transport system (75), and to the analog azaserine, which is transported by both the glutamine and histidine transport systems (Table 3, footnote d), suggest that glutamine transport is elevated.

(iii) Their faster growth rate on arginine as nitrogen source suggests that arginine transport is elevated. (The interpretation that faster growth is due at least in part to increased transport is based on the observation that mutants and wild type have the same very fast growth rate [doubling time ~50 min] on the dipeptide arginylarginine; this peptide is rapidly transported, probably by a dipeptide transport system [14]. Presuming that the dipeptide is catabolized by the same enzymes as arginine after it is hydrolyzed, both wild type and the mutant strains are capable of equally rapid arginine catabolism.)

TABLE 3. *Growth characteristics[a] of wild-type and N-regulatory mutant strains*

Compound tested	Wild type	Regulatory mutant
D-Histidine growth[b]	No growth (>11 h)	91 min
HIPA inhibition[c]	2.3	3.3
Azaserine inhibition[d]	0.9	2.1, clear
γ-Glutamylhydrazide inhibition[e]	1.5	3.1, clear
Arginine growth[f]	738 min	102 min
Arginylarginine growth[f]	51 min	55 min
Ornithine growth[g]	>12 h	166 min
Glutamate growth[g]	~169 min	~135 min
Aspartate growth[g]	~145 min	~120 min
α-Methyl glutamate inhibition[h]	Resistant	1.3, clear
D-Glutamate inhibition[i]	Resistant	3.0

[a] Ability to grow on D-histidine as histidine source or on arginine, arginylarginine, ornithine, glutamate, or aspartate as nitrogen source is expressed as doubling time at 37°C. The wild-type strain for growth experiments was TA831 and the N-regulatory mutant was SK214. Inhibition by amino acid analogs is expressed as the diameter of the inhibition zone (in centimeters) around a disk containing the analog. The wild-type strain for inhibition studies was TA1772 and the N-regulatory mutant was TA3290, except as noted. (The diameter of the disks was 0.6 cm.) Strain SK214 had the same doubling time as wild-type on cytosine, cytidine, or adenosine as nitrogen source and did not have increased sensitivity to 5-fluorocytosine. Strains SK214 and TA3290 failed to grow on proline as nitrogen source, an observation that was not pursued. These strains did not acquire the ability to grow on arginine, glutamate, aspartate, or glutamine as sole carbon source.

[b] 0.3 mM D-histidine (the nitrogen source was NH_4^+). Doubling times on L-histidine were 54 and 58 min, respectively, for TA831 and SK214.

[c] Inhibition at 1 μmol.

[d] Inhibition at 20 nmol. Phenylalanine at a final concentration of 0.7 mM was added to the plates to block transport of azaserine through the aromatic permease (8). Increased azaserine sensitivity in the regulatory mutant is apparently mediated by increased transport through both histidine and glutamine transport systems, since both amino acids must be present to completely reverse the inhibition.

[e] Inhibition at 2 μmol. The wild-type strain was TA831 and the N-regulatory mutant strain was SK214.

[f] Growth at 5 mM arginine or 2.5 mM arginylarginine. The carbon source was glucose (0.4%). As the strains grow on arginylarginine the dipeptide disappears from the medium and free arginine appears, as indicated by amino acid analysis. After 3 to 4 doublings (optical density at 650 nm of ~0.5), no dipeptide remains in the medium and each strain shifts to a doubling time characteristic of its growth on arginine. Doubling times on NH_4^+ as nitrogen source were 54 and 58 min, respectively, for TA831 and SK214.

[g] Growth at 10 mM ornithine, glutamate, or aspartate with glucose as carbon source.

[h] Inhibition at 1.2 μmol.

[i] Inhibition at 3.5 μmol.

(iv) Their slightly improved growth rate on glutamate or aspartate as nitrogen source and their greatly increased sensitivity to the analogs α-methylglutamate (66) and D-glutamate, suggest that glutamate transport is elevated.

Genetic analysis of N-regulatory mutations. The N-regulatory mutations we have studied are very closely linked to the structural gene for glutamine synthetase, *glnA* (43), at 86 units on the chromosomal map (65). The *gln-128* and *gln-139* mutations were 100% linked by P22-mediated transduction to the Δ(*glnA-glnR*)*60* mutation (100 out of 100 transductants tested in each case). Similar results were obtained for the *gln-125-gln-127* and *gln-481-gln-483* mutations; in these cases only a few transductants were tested.

The following observations support the conclusion that the N-regulatory mutations we have analyzed are likely to be single mutations. (i) They occurred spontaneously at high frequency (see Materials and Methods). (ii) All properties of N-regulatory mutant strains could be transferred by transduction to appropriate recipients (e.g., the properties of strains SK214 and SK295 were essentially the same as those of strains SK210 and TA3173, respectively). (iii) Spontaneous revertants that were isolated from several N-regulatory mutant strains (including SK214 and TA3173) by selecting for resistance to γ-glutamylhydrazide or HIPA had regained a wild-type phenotype with respect to D-histidine and arginine growth also. These results indicate that a single mutation is responsible for the pleiotropic phenotype of N-regulatory mutant strains.

Transport of histidine and glutamine in N-regulatory mutant strains. We have focused on transport of histidine and glutamine for the following reasons. (i) The transport of histidine has been characterized extensively, and conditions are known for assaying specifically the high-affinity histidine permease (3, 12), of which the histidine-binding protein (J protein) is a component (7, 8). (ii) A single high-affinity glutamine transport system is known in *E. coli* and the glutamine-binding protein is a component of this system (75).

The levels of histidine transport in wild type and the N-regulatory mutant strain TA3290 (*gln-139*) grown under conditions of nitrogen excess are shown in Fig. 1; at 1.5 nM, histidine strain TA3290 has 2.5 times more transport activity than wild type. A Lineweaver-Burk plot of

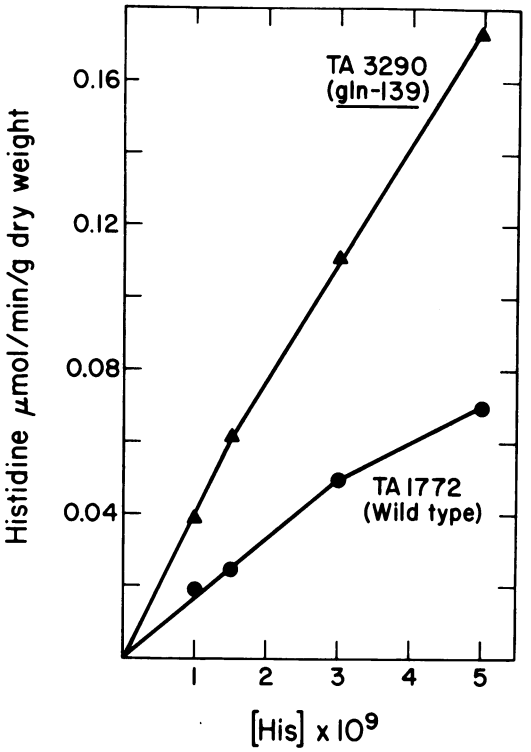

FIG. 1. *Transport of L-histidine (1 through 5 nM) through the high-affinity histidine transport system in N-regulatory mutant strain TA3290 (gln-139) and wild-type, TA1772. Assay conditions are described in Materials and Methods. Rates are expressed in micromoles per minute per gram dry weight.*

the data in Fig. 1 indicates that V_{max} for strain TA3290 is 4.2 times that of wild type, whereas the apparent affinities of the two strains for histidine are the same (apparent K_m, 14 nM). These results indicate that the normal components of the high-affinity histidine transport system are present in elevated amounts in the N-regulatory mutant strain.

The levels of glutamine transport in the N-regulatory mutant strain SK214 (*gln-128*) and wild-type strain TA831 grown under conditions of nitrogen excess are shown in Fig. 2: at 8 μM glutamine strain SK214 has 3.5 times more transport activity than strain TA831. Studies of mutant strains that lack the glutamine-binding protein (see below) demonstrate that the binding protein is an essential component of the glutamine transport system that is elevated in strain SK214.

Binding activities. The following results demonstrate that synthesis of several periplasmic transport components is elevated in N-regulatory mutant strains and can be elevated physiologically in the wild-type strain by nitrogen limitation. Binding activities in periplasmic fractions of wild-type and N-regulatory mutant strains were measured by equilibrium dialysis. Binding activities for histidine, glutamine, arginine, and glutamate in the N-regulatory mutant strain SK214 were 2.6-, 13-, 6-, and 4.3-fold higher than those of wild type, respectively, when cells were grown with excess nitrogen (Table 4). (Similar results were obtained for N-regulatory mutant strain TA3239.) As far as is known, under the conditions we have used for growth of cells and assays of binding, the binding activities for histidine (42), glutamine (75, 78), and glutamate (1, 15, 76) are due to a single protein each, whereas that for arginine is due to three proteins (62, 79). Immunological studies described below indicate that increases in histidine-, glutamine-, and glutamate-binding activity are due to increases in the amounts of the known binding proteins. As shown in Table 4, the increase in arginine binding appears to be due largely to an increase in the LAO-binding protein; this is demonstrated by the fact that increased arginine binding is inhibited by lysine (62). The elevations in arginine binding by LAO and non-LAO proteins are 36- and 2.4-fold, respectively.

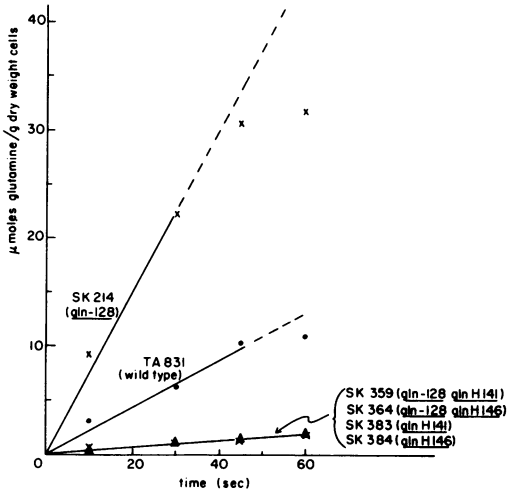

FIG. 2. *Transport of glutamine (8 μM) by N-regulatory mutant strain SK214 (gln-128 hisF645), the isogenic wild-type strain TA831 (hisF645), and strains lacking the activity of the glutamine-binding protein SK359 (gln-128 glnH141 hisF645), SK364 (gln-128 glnH146 hisF645), SK383 (glnH141 hisF645 zig-205::Tn10) and SK384 (glnH146 hisF645 zig-205::Tn10). Assay conditions are described in Materials and Methods. Rates of transport (micromoles per minute per gram dry weight) were 13 for TA831, 45.3 for SK214 and 1.8 to 2.2 for strains lacking activity of the glutamine-binding protein.*

TABLE 4. *Binding activities[a] in periplasmic fractions of wild-type and an N-regulatory mutant strain*

Growth condition	Strain	pmol bound/mg (dry weight) of cells[b]				
		Histidine	Glutamine	Total arginine	LAO[c]	Glutamate
Excess nitrogen[d]	Wild-type TA831	10 (1)[f]	4.5 (1)	13.8 (1)	1.5 (1)	0.3 (1)
	N-regulatory mutant SK214	26 (2.6)	58.4 (13)	83.6 (6)	54.3 (36)	1.3 (4.3)
Limiting nitrogen[e]	Wild-type TA831	17 (1.7)	14.2 (3.2)	36.7 (2.7)	23.7 (16)	0.8 (2.7)
	N-regulatory mutant SK214	47 (4.7)	71.7 (16)	165.3 (12)	131 (87)	2.5 (8.3)

[a] Binding activities in periplasmic fractions (0.3-ml samples) were assayed by equilibrium dialysis at a free amino acid concentration of 10 nM. Shock fluids of strain TA831 (0.32 mg of protein/ml) were diluted 5-fold, 5-fold, and 10- to 20-fold for assays of glutamine, histidine, and arginine binding, respectively, and were not diluted for assays of glutamate binding. Shock fluids of strain SK214 (0.38 mg of protein/ml) were diluted about 4-fold more than those of strain TA831 in each case, except that they were not diluted for assays of glutamate binding.

[b] Activity is expressed as picomoles of amino acid bound relative to the amount of cells from which the periplasmic fraction was prepared. A conversion factor of 0.47 mg (dry weight) of cells/ml at an optical density at 650 nm of 1.0 was used. For determination of histidine-binding activity of wild type the shock fluid obtained from approximately 1 mg (dry weight) of cells was used for assay.

[c] Binding that was inhibited by 1 μM L-lysine.

[d] Minimal medium with 10 mM NH_4^+ and 3 mM glutamine as nitrogen sources.

[e] Minimal medium with 10 mM glutamate as nitrogen source.

[f] Numbers in parentheses are fold-elevation relative to a value of 1 for wild type under conditions of nitrogen excess.

Binding activities for the four amino acids of interest were elevated physiologically in the wild-type strain by limitation of nitrogen: the elevations in binding activity for histidine, glutamine, arginine (total), arginine (LAO), and glutamate were 1.7-, 3.2-, 2.7-, 16-, and 2.7-fold, respectively (there was no detectable elevation in arginine binding by non-LAO proteins). When the N-regulatory mutant strain SK214 was grown under nitrogen-limiting conditions, it had even higher binding activities than under conditions of nitrogen excess (Table 4); activities for the mutant strain were higher than those for wild type under identical nitrogen-limiting conditions.

A control experiment indicated that there was no elevation in leucine binding in periplasmic fractions of strain SK214 and that leucine binding was not elevated physiologically in either strain TA831 or strain SK214 by nitrogen limitation (data not shown).

Levels of binding protein antigens. Elevations of binding proteins in N-regulatory mutant strains and in wild-type strains under nitrogen-limiting growth conditions were confirmed immunologically by Ouchterlony double-diffusion tests as described in Materials and Methods. The N-regulatory mutant strain SK214 contained more than 16 times as much antigen as the wild-type strain TA831 for the LAO-binding protein and about 12, 4, and 2 times as much for the glutamine-, glutamate-, and histidine-binding proteins, respectively, when both strains were grown with excess nitrogen. (Since the LAO-binding protein could not be detected in extracts of strain TA831 grown with excess nitrogen, we can give only a minimum estimate of the elevation of this protein in strain SK214.) The elevations in binding protein antigens are in agreement with those obtained by direct assays of binding activity. Both strains TA831 and SK214 contained higher concentrations of all binding protein antigens under nitrogen-limiting growth conditions than under nitrogen excess conditions (data not given). The LAO-binding protein was easily detected at a 1/8 dilution of extracts of strain TA831 grown under nitrogen-limiting conditions.

Amounts of binding protein bands after SDS-PAGE. Periplasmic fractions from wild-type and N-regulatory mutant strains were analyzed by SDS-PAGE. As shown in Fig. 3, a periplasmic fraction from the N-regulatory mutant strain SK214 (well 3) contains increased amounts of several protein bands relative to the same fraction from wild type (well 1) when both strains are grown with excess nitrogen. (Similar results were obtained for N-regulatory mutant strain TA3239 [data not shown].) The same bands that are elevated in N-regulatory mutant strains are elevated in wild type by limitation of nitrogen (well 2) and are elevated even further in N-regulatory mutant strains under these conditions (well 4). Since the four proteins of interest fail to bind tightly to DEAE-cellulose at neutral pH, changes in the levels of these proteins are more clearly visible after removal of most contaminating proteins by adsorption to

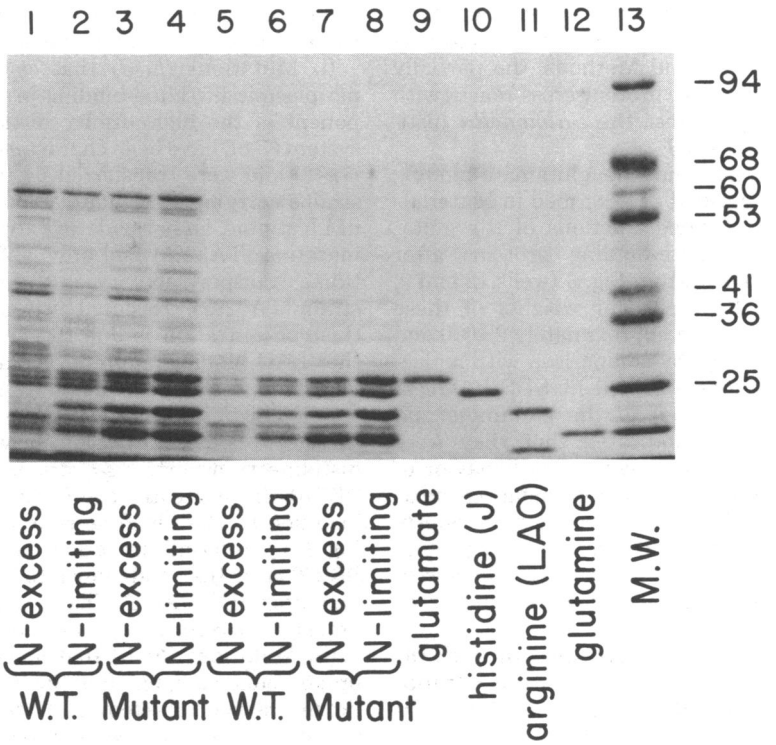

FIG. 3. *SDS-PAGE (9%) of periplasmic fractions (~2 μg protein in each case) from TA831 (wells 1 and 2), N-regulatory mutant strain SK214 (wells 3 and 4), and the same fractions after removal of contaminating proteins by adsorption to DEAE-cellulose (wells 5 through 8). The nitrogen sources were 10 mM NH_4Cl plus 3 mM glutamine for nitrogen excess and 10 mM glutamate for nitrogen limitation. In both cases glucose (0.4%) was the carbon source. Wells 9 through 12 contained, respectively: purified glutamate-binding protein, purified histidine-binding protein J, partially purified LAO protein (top band), purified glutamine-binding protein (~0.15 μg of each). Well 13 contained the following molecular weight standards (the numbers in parentheses are the respective subunit molecular weights as in reference 9): phosphorylase b (94,000), bovine serum albumin (68,000), beef liver catalase (60,000), bovine liver glutamic dehydrogenase (53,000), horse liver alcohol dehydrogenase (41,000), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36,000), Salmonella histidine-binding protein J (25,000); Salmonella glutamine-binding protein was also present. Since there were no protein bands of molecular weight higher than phosphorylase b, the top portion of the gel is not included in the figure.*

DEAE-cellulose (wells 5 through 8). We have identified the four protein bands (see below) as a glutamate-binding protein, the histidine-binding protein J, the LAO-binding protein, and a glutamine-binding protein, in order of decreasing molecular weight. Purified or partially purified preparations of the individual proteins have been run on the same gel for comparison (wells 9 through 12).

Purification of binding proteins. The purpose of these studies was to correlate the increases in binding activities in N-regulatory mutant strains and in wild-type strains under nitrogen-limiting conditions with specific binding proteins and specific bands on SDS-PAGE.

The histidine-binding protein J has been purified and extensively characterized (44). A sample of this protein after SDS-PAGE is shown in Fig. 3 (well 10).

An arginine-binding protein (LAO protein) was partially purified as described in Materials and Methods. A sample of the partially purified protein after SDS-PAGE is shown in Fig. 3 (well 11): the molecular weight of the protein (upper band in well 11) appears to be slightly less than that of the histidine-binding protein J, which is 25,000 (44). Binding of [^{3}H]arginine by the partially purified arginine-binding protein (at a free arginine concentraton of 10 nM) was 95% inhibited by unlabeled lysine (at 1 μM). In addition, the chromatographic properties of this arginine-binding protein are similar to those of the LAO-binding protein from *E. coli* (62). On these bases we have identified this protein as the LAO-

binding protein. (There are two other arginine-binding proteins known in *E. coli* [62, 63].) As noted in Materials and Methods, the partially purified LAO-binding protein cross-reacts with antibody raised against the *Salmonella* histidine-binding protein J.

The glutamine- and the glutamate-binding proteins were purified as described in Materials and Methods. Purified fractions of the glutamine- and glutamate-binding proteins after SDS-PAGE are shown in Fig. 3 (wells 12 and 9, respectively); the molecular weights of these proteins appear to be approximately 23,000 and 30,000, respectively, by comparison with molecular weight standards in well 13. SDS-PAGE of large amounts (~3 μg) of the glutamine and glutamate proteins indicated that they were >95% pure (data not shown). The behavior of each of these proteins on ion-exchange chromatography is similar to that reported previously (1, 78) and to that of the corresponding protein from *E. coli* (15, 75, 76). Each cross-reacts with antiserum raised against the corresponding *E. coli* protein.

N-regulatory mutant strains that lack individual binding proteins or other components of individual transport systems. N-regulatory mutant strains that lack components of individual transport systems were isolated to determine the roles of individual transport components in the phenotype of N-regulatory mutant strains and in the elevations of transport observed in such strains, and to demonstrate that the structural genes for various transport components were not clustered on the chromosome and that regulatory effects were therefore exerted on several unlinked genes.

(i) Mutations (*hisJ*) that cause loss of the periplasmic histidine-binding protein J, a component of the high-affinity histidine transport system (7, 8), have been characterized previously (7, 42) and were transferred by transduction to strains carrying N-regulatory mutations. At 1.5 nM histidine, an N-regulatory strain with a *hisJ* mutation (TA3285) had only 33% as much histidine transport activity as the isogenic *hisJ*$^+$ strain (TA3292). (Residual histidine transport at this concentration is due to elevated levels of the LAO-binding protein [data not shown] which are also responsible for a small amount of residual histidine binding activity in periplasmic fractions [Table 5].) Consistent with decreased histidine transport, *hisJ* strains (TA3174 and SK270) have greatly decreased growth on D-histidine as the histidine source. As shown in Fig. 4, a periplasmic fraction from the *hisJ* strain SK270 lacks the band known to be the J protein (42) after SDS-PAGE. The *hisJ* gene lies at 49 units on the *Salmonella* chromosome (65).

(ii) Mutations (*hisP*) that inactivate a membrane-bound component of the high-affinity histidine transport system (10) were also transferred to strains carrying N-regulatory mutations by transduction. Regulatory strains carrying a *hisP* mutation (e.g., TA3294, SK425, and SK426) lost the ability to grow on D-histidine and became resistant to the histidine analog HIPA. In agreement with the fact that *hisP* function is known to be required for transport of arginine when this amino acid is used as nitrogen

TABLE 5. *Binding activities*[a] *of N-regulatory mutant strains that lack individual binding proteins*

Strain	Relevant genotype	% of activity in strain SK214[b]			
		Histidine	Arginine	LAO[c]	Glutamine
SK214	*gln-128*	100	100	100	100
TA3292[d]	*gln-139 dhuA1*	590	148	109	96
SK270	*gln-128 dhuA1 hisJ5601*	3[e]			100
TA3293	*gln-139 dhuA1 argT526*		63	9	108
SK359	*gln-128 glnH141*	190			1
SK364	*gln-128 glnH146*	130	130		3
SK367	*gln-128 glnH149*				<1
SK369	*gln-128 glnH151*				2

[a] Binding activities in periplasmic fractions (0.3 ml samples) were assayed by equilibrium dialysis at a free amino acid concentration of 10 nM and were calculated as picomoles of amino acid bound relative to the amount of cells from which the periplasmic fraction was prepared. Values for strain SK214 were set at 100%. Cells were grown with excess nitrogen. Blank space means not determined.

[b] Binding activities for strain SK214 were 11, 69, 47, and 24 pmol/mg (dry weight) of cells for histidine, arginine, LAO, and glutamine, respectively.

[c] Fraction of total arginine binding that was inhibited by 1 μM lysine.

[d] TA3292 is isogenic with the *argT* strain TA3293.

[e] A strain without the regulatory mutation *gln-139* has no detectable histidine binding since the histidine-binding protein J is not produced in strains carrying the *hisJ5601* mutation (42). Residual histidine binding is probably due to elevated levels of the LAO protein that result from the regulatory mutation.

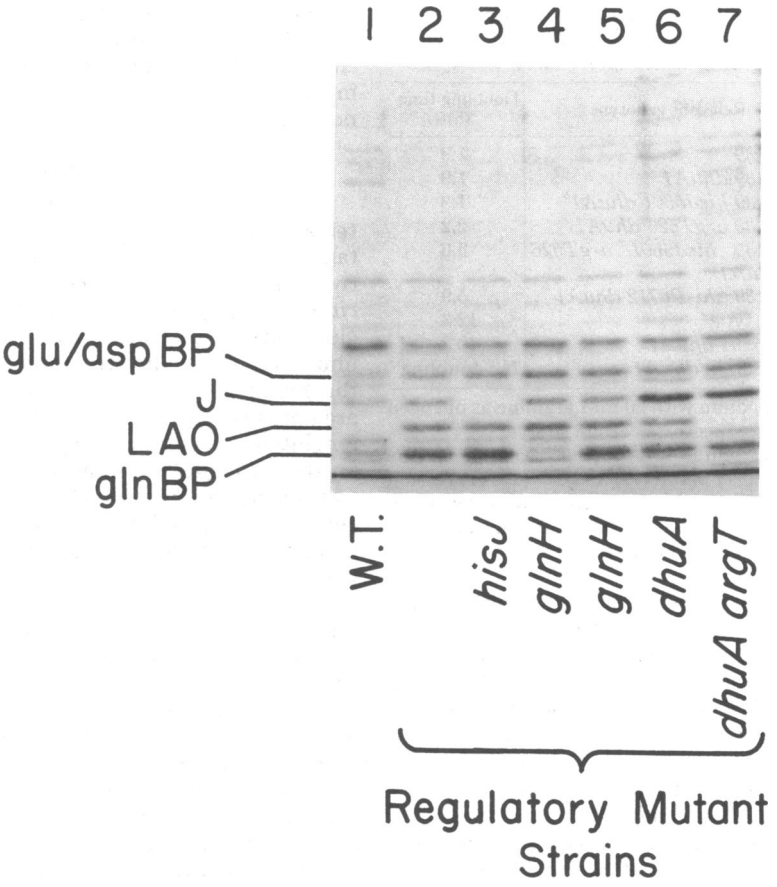

FIG. 4. *SDS-PAGE (9%) of periplasmic fractions (~1.2 μg of protein) from TA831 (well 1), N-regulatory mutant strain SK214 (well 2), and N-regulatory mutant strains carrying the following additional mutations: dhuA1 hisJ5601 (SK270, well 3), glnH146 (SK364, well 4), glnH141 (SK359, well 5), dhuA1 (TA3292, well 6), and dhuA1 argT526 (TA3293, well 7). The strains in wells 3, 4, and 7 lack the histidine-, glutamine-, and LAO-binding proteins, respectively, and the strain in well 5 produces an inactive glutamine binding protein. The strains in wells 3, 6, and 7 carry a mutation in the promoter for the high-affinity histidine transport operon dhuA1 which elevates synthesis of the histidine-binding protein J above that seen in the N-regulatory mutant. All periplasmic fractions had been treated with DEAE-cellulose to remove proteins other than those of interest, as described in Materials and Methods. Since the proteins of interest are of low molecular weight, only the bottom portion of the gel is shown in the figure.*

source (41), N-regulatory mutant strains carrying a *hisP* mutation lost the ability to grow rapidly on arginine as nitrogen source (Table 6). The fact that they retained ability to grow on arginine about twice as fast as wild type suggests that N-regulatory mutations elevate transport of arginine through a system(s) other than those that involve *hisP*, as well as through *hisP* systems.

(iii) A mutation (*argT*) that caused loss of the LAO-binding protein was isolated in a strain with an N-regulatory mutation as described in Materials and Methods. As shown in Fig. 4, an *argT* strain (TA3293) lacks the band identified as the LAO protein on SDS-PAGE. This strain showed a 60% decrease in total arginine binding relative to an isogenic $argT^+$ strain, TA3292 (Table 5). (Consistent with this, arginine binding in the $argT^+$ strain was 50% inhibited by lysine.) Residual arginine binding in the *argT* strain was no longer inhibited by lysine and is presumed to be due to other arginine-binding proteins (62, 63, 79). The *argT* strain TA3293 grew almost as rapidly on arginine as nitrogen source as the isogenic $argT^+$ strain TA3292 (Table 6). However, an N-regulatory mutant strain that lacked both the histidine and LAO-binding proteins, TA3298, lost the ability to grow rapidly on arginine, like a strain that lacked *hisP* function (Table 6). This suggests that the membrane-

TABLE 6. *Growth of N-regulatory mutant strains that lack individual transport components on arginine as nitrogen source*[a]

Strain	Relevant genotype	Doubling time (h)
TA3290	*gln-139*	1.9
TA3292	*gln-139 dhuA1*	1.9
TA3285	*gln-139 hisJ5601 dhuA1*[b]	1.9
TA3293	*gln-139 argT526 dhuA1*	2.2
TA3298	*gln-139 hisJ5601 argT526 dhuA1*	5.6
TA3294	*gln-139 ΔhisP6712 dhuA1*	5.9
TA1772	Wild-type	12.3
TA1650	*hisJ5601 dhuA1*	10.3
SK427	*ΔhisP6712 dhuA1*	No doubling

[a] Minimal medium with 20 mM arginine as nitrogen source and 0.4% glucose as carbon source.

[b] The *dhuA1* mutation is in the promoter for the histidine transport operon (11) and elevates expression of the operon (7, 40). *hisJ* mutations have been isolated only in strains that carry the *dhuA1* mutation and have not been separated from it.

bound *hisP* protein must function together with either the LAO-binding protein or the J protein in the transport of arginine. (A strain that lacked only the J protein showed no growth defect on arginine [Table 6].) The *argT* mutation is very closely linked (98% by P22-mediated transduction) to the operator end of the histidine transport operon but is not within the operon (G. F.-L. Ames, unpublished data).

(iv) Spontaneous mutations (*glnH*) that cause loss of the glutamine-binding protein or loss of its binding activity were isolated in a strain with an N-regulatory mutation, SK214, as described in Materials and Methods. At 8 μM glutamine, two strains with *glnH* mutations, SK359 and SK364, had only 4% as much glutamine transport activity as their parent strain SK214 (Fig. 2). Shock fluids from these strains and two additional *glnH* strains had <3% as much glutamine-binding activity as their parent (Table 5). Strain SK364 lacks glutamine-binding protein antigen as well as binding activity, whereas strain SK359 lacks activity but has the antigen and thus has a mutation in the structural gene for the glutamine-binding protein. Periplasmic fractions of these strains after SDS-PAGE are shown in Fig. 4. As expected, strain SK364 lacks a band at the position of the glutamine-binding protein, whereas strain SK359 has as much of this band as its parent. The growth rates of strains SK359 and SK364 on glutamine as nitrogen source were only slightly decreased from that of their parent (data not shown). Mutations in these strains were not linked to *glnA*, the structural gene for glutamine synthetase (43), by P22-mediated transduction (0 out of 100 γ-glutamyl hydrazide resistant), nor were they linked to *hisP* (0 out of 100 γ-glutamyl hydrazide sensitive). Linkage of *glnH* to a gene known to be involved in glutamine transport, *glnP* (20), has not been determined.

DISCUSSION

Synthesis of four periplasmic transport proteins, which bind the amino acids histidine, glutamine, LAO, and glutamate-aspartate, respectively, is under nitrogen control in *S. typhimurium*; synthesis of these proteins, like that of glutamine synthetase, is increased by nitrogen limitation. Since the structural genes encoding the histidine- and glutamine-binding proteins are not clustered with *glnA*, the structural gene for glutamine synthetase (43), or with each other on the *Salmonella* chromosome, nitrogen control affects the expression of several unlinked genes. This excludes the possibility that such regulation is limited to genes in the same operon with *glnA*.

There is currently a major interest in determining the mechanism(s) for nitrogen control of the synthesis of various bacterial proteins including glutamine synthetase, amino acid degradative enzymes, nitrogenase and other proteins required for nitrogen fixation, and sporulation-specific proteins in *Bacillus*. We are attempting to determine the mechanism for nitrogen control of the synthesis of glutamine synthetase and transport proteins in *Salmonella*. Magasanik and Tyler and their colleagues have proposed that glutamine synthetase itself mediates nitrogen control in the related enteric bacteria *K. aerogenes* and *E. coli*; they propose that glutamine synthetase directly controls its own synthesis in response to the availability of nitrogen (autogenous regulation) (17, 29; reviewed in 46, 47, 72) and that it plays a major role in controlling synthesis of other enzymes under nitrogen control (54, 55, 73; reviewed in 46, 47, 72). Our previous findings have led us to question the view that glutamine synthetase controls its own synthesis. First, we demonstrated that the product of the *glnF* gene, which is well separated from *glnA* on the chromosome, is essential for synthesis of glutamine synthetase in *Salmonella* and probably *E. coli* (33). The *glnF* gene was subsequently identified in *Klebsiella* as well (32). The existence of such a positive regulatory gene, which could not have been predicted from the Magasanik model (46, 47), precludes purely autogenous regulation of the synthesis of glutamine synthetase (33). Second, contrary to Magasanik and his colleagues (29, 36, 55, reviewed in 46), we found that altering the degree of covalent modification of glutamine

synthetase by mutation did not significantly affect regulation of its synthesis (13).

One of the lines of evidence presented by Magasanik and Tyler and their colleagues to support their view that glutamine synthetase is a genetic regulatory element is the following: mutations in *K. aerogenes* and *E. coli* that result in constitutive high-level synthesis of glutamine synthetase and other enzymes under nitrogen control (GlnC phenotype [23, 54, 55]) were found to lie within *glnA* (16, 17, 54, 70), although no change in the glutamine synthetase protein from such a strain was demonstrated (16). Although we do not know the precise location of analogous mutations (the N-regulatory mutations described in this paper) on the *Salmonella* chromosome, we favor the hypothesis that they lie in a regulatory gene separate from *glnA*. We have recently identified a gene encoding a positive regulatory factor, *glnR*, that is very close to the *glnA* gene on the *Salmonella* chromosome (S. Kustu, E. Garcia, S. Bancroft, N. McFarland, S. Hui, and D. Burton, manuscript in preparation). Mutations that lead to complete loss of function of the *glnR* gene product (not those described in this paper) result in synthesis of low fixed levels of glutamine synthetase and periplasmic transport proteins, regardless of the availability of nitrogen (Kustu et al., manuscript in preparation). We are currently testing the hypothesis that the N-regulatory mutations described in this paper, which result in synthesis of high levels of the same proteins, also lie within *glnR* and result in formation of an altered *glnR* gene product. We think the possibility should be considered that all similar mutations (GlnC-type) in the enteric bacteria (*K. aerogenes* [23, 55], *E. coli* [54], and *S. typhimurium* [31]) lie within the *glnR* gene rather than *glnA*.

We have demonstrated that synthesis of several amino acid transport systems is subject to general control by availability of nitrogen in the growth medium. This is analogous to general control by availability of carbon and energy, which has been demonstrated for the major proline permease of *Salmonella* (60) and the aromatic permease (2).

In addition to direct evidence that synthesis of soluble, periplasmic transport components is under nitrogen control, we have some evidence that synthesis of membrane-bound components is similarly regulated. Genetic studies of transport systems with periplasmic-binding protein components, e.g., those for histidine and leucine, have indicated that periplasmic proteins cannot function by themselves in transport (7, 53). For example, the histidine-binding protein J must function together with at least one additional protein, the product of the *hisP* gene (7), which was recently demonstrated to be an intrinsic cytoplasmic membrane protein (10). The N-regulatory mutant strains described in this paper, which elevate synthesis of the J protein, also elevate synthesis of the P protein (10); thus synthesis of both components of the high-affinity histidine transport system is subject to nitrogen control. (This was expected since the genes for these components, *hisJ* and *hisP*, are known to be part of the same operon [11].) By analogy with the histidine system, the other amino acid transport systems we have studied presumably have membrane-bound as well as periplasmic components; synthesis of the membrane-bound components may also be under nitrogen control.

The phenotype of N-regulatory mutant strains suggests that synthesis of amino acid transport systems without periplasmic components is under nitrogen control in *Salmonella*. For example, N-regulatory mutant strains have increased sensitivity to the glutamate analog α-methylglutamate, which is transported in *E. coli* by a Na^+-dependent glutamate transport system that is distinct from the glutamate system with a periplasmic-binding protein component (66). (This membrane-bound system, unlike systems with periplasmic components, is active in membrane vesicles [37].)

The significance of the pattern of transport systems that is under nitrogen control in *S. typhimurium* is not known. Under nitrogen-limiting conditions, synthesis of high-affinity amino acid transport systems may be elevated to scavenge amino acids from the growth medium for protein synthesis (4). Alternatively, the high-affinity transport systems and other lower-affinity systems without periplasmic components may function in transport of amino acids for use as a source of nitrogen (4) (glutamine, arginine, glutamate, and aspartate will serve as the sole nitrogen source for *S. typhimurium* LT2).

Although *Salmonella* has retained nitrogen control of amino acid transport systems, it has apparently lost nitrogen control of the synthesis of several amino acid degradative enzymes (e.g., those for histidine and proline [24, 28, 51, 56]). Since N-regulatory mutant strains of *Salmonella* grow rapidly on arginine as nitrogen source, it is possible that *Salmonella* has retained nitrogen control of the arginine degradative enzymes. The pathway for arginine catabolism in *K. aerogenes* has recently been elegantly elucidated by Friedrich and Magasanik (30) who showed that degradation is initiated by a novel amidinotransferase; if the same pathway pertains in *Salmonella*, it should be a straightforward matter to test for nitrogen control of the arginine catabolic enzymes.

Nitrogen control of amino acid transport oc-

curs in enteric bacteria other than *Salmonella*. Willis et al. demonstrated nitrogen control of high-affinity glutamine transport in *E. coli* (77) as did Betteridge and Ayling in *Salmonella* (21), although neither group measured levels of the glutamine-binding protein. In agreement with the above, regulatory mutant strains (GlnC-type) of *E. coli* similar to the N-regulatory mutant strains we have described have increased sensitivity to the glutamine analog L-methionine-DL-sulfoximine (54); Pahel et al. suggested that sensitivity was due to increased glutamine transport since glutamine synthetase from such a strain did not show altered sensitivity to the analog (54). (Betteridge and Ayling demonstrated that methionine sulfoximine is transported by the high-affinity glutamine transport system in *S. typhimurium* [20].) It is interesting that nitrogen control of the synthesis of transport systems, particularly of general amino acid transport systems, apparently extends to a variety of fungi; these include *Penicillium* (18, 19), *Neurospora* (38), and *Saccharomyces cerevisiae* (34, 61).

ACKNOWLEDGMENTS

We thank Elena Spudich for isolating some of the N-regulatory mutant strains, D. Biek for helping to purify the LAO protein, and C. E. Furlong for antisera and many helpful suggestions. While this work was in progress, related studies were performed in Jean Brenchley's laboratory, and we thank her for discussing them with us before publication. Finally, we thank L. N. Csonka, J. L. Ingraham, and L. L. McCarter for thoughtful criticisms of the manuscript.

This work was supported by Public Health Sevice grants GM21307 and AM12121 to S.K. and G.A., respectively. During the period of this work N.C.M. was supported by Public Health Service Training Grant GM1041.

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