

# Simultaneous Prevention of Glutamine Synthesis and High-Affinity Transport Attenuates *Salmonella typhimurium* Virulence

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In *Salmonella typhimurium*, transcription of the *glnA* gene (encoding glutamine synthetase) is under the control of the nitrogen-regulatory (*ntr*) system comprising the alternate sigma factor  $\sigma^{54}$  (NtrA) and the two-component sensor-transcriptional activator pair NtrB and NtrC. The *glnA*, *ntrB*, and *ntrC* genes form an operon. We measured the virulence of *S. typhimurium* strains with nitrogen-regulatory mutations after intraperitoneal (i.p.) or oral inoculations of BALB/c mice. Strains with single mutations in *glnA*, *ntrA*, *ntrB*, or *ntrC* had i.p. 50% lethal doses (LD<sub>50</sub>s) of <10 bacteria, similar to the wild-type strain. However, a strain with a  $\Delta(glnA-ntrC)$  operon deletion had an i.p. LD<sub>50</sub> of >10<sup>5</sup> bacteria, as did  $\Delta glnA ntrA$  and  $\Delta glnA ntrC$  strains, suggesting that *glnA* strains require an *ntr*-transcribed gene for full virulence. High-level transcription of the glutamine transport operon (*glnHPQ*) is dependent upon both *ntrA* and *ntrC*, as determined by *glnHP-lacZ* fusion measurements. Moreover,  $\Delta glnA glnH$  and  $\Delta glnA glnQ$  strains are attenuated, similar to  $\Delta glnA ntrA$  and  $\Delta glnA ntrC$  strains. These results reveal that access of *S. typhimurium* to host glutamine depends on the *ntr* system, which apparently is required for the transcription of the glutamine transport genes. The  $\Delta(glnA-ntrC)$  strain exhibited a reduced ability to survive within the macrophage cell line J774, identifying a potential host environment with low levels of glutamine. Finally, the  $\Delta(glnA-ntrC)$  strain, when inoculated at doses as low as 10 organisms, provided mice with protective immunity against challenge by the wild-type strain, demonstrating its potential use as a live vaccine.

Glutamate and glutamine serve as the primary nitrogen donors for all cellular metabolites in *Salmonella typhimurium* and other enteric bacteria (for a review, see reference 51). The enzyme glutamine synthetase (encoded by the *glnA* gene) is the sole means for synthesis of glutamine. Additionally, glutamine synthetase is responsible for all ammonia assimilation under conditions of low external nitrogen concentrations (57). Both the synthesis and the activity of glutamine synthetase are regulated in response to the availability of nitrogen.

Transcription of *glnA* is under the control of the nitrogen-regulatory (*ntr*) system (for a review, see reference 49). The major promoter (P2) (Fig. 1) is recognized by an alternate holoenzyme form of RNA polymerase containing  $\sigma^{54}$  (encoded by the *ntrA* gene), which requires an activator protein to form transcriptionally active open promoter complexes (19, 54). The NtrC protein is a  $\sigma^{54}$  transcriptional activator; it binds cooperatively to two upstream sites and activates transcription under nitrogen-limiting conditions by directly contacting  $\sigma^{54}$ -holoenzyme bound at the P2 promoter (48, 50, 52, 60). NtrC must be phosphorylated to activate transcription; the NtrB protein causes phosphorylated NtrC (NtrC-P) to be formed only under nitrogen-limiting conditions (24, 43). The *ntrB* and *ntrC* genes lie downstream within the *glnA* operon (39). A secondary  $\sigma^{70}$ -dependent promoter (P1) (Fig. 1) lies between the two NtrC binding sites; the binding of NtrC at these two sites represses transcription from P1 (38, 46).

The high-affinity glutamine transport system, encoded by the *glnHPQ* operon, is also under the control of the *ntr* system: levels of the glutamine periplasmic binding protein (GlnH) are coordinately regulated with levels of glutamine synthetase (4,

31, 61), as well as those of transport proteins for glutamate-aspartate, histidine, and lysine-arginine-ornithine (31). In *Escherichia coli*, the major *glnH* promoter has been shown to be transcribed by  $\sigma^{54}$ -holoenzyme and NtrC in vitro. At this promoter, contact between NtrC-P and  $\sigma^{54}$ -holoenzyme is enhanced by the binding of integration host factor (IHF) at a site between the NtrC sites and the promoter (6).

*S. typhimurium* is a bacterial pathogen which causes gastroenteritis in humans (for a review, see reference 14). In mice, oral administration leads to a lethal systemic disease and serves as an animal model for human typhoid fever caused by *Salmonella typhi*. After being ingested, salmonellae typically invade enterocytes of the small intestine, transcytose through the epithelium, and enter the lamina propria, where they are phagocytosed by macrophages (5). Salmonellae survive within macrophages and are transported to the liver and spleen, where they replicate and eventually cause systemic infection. Little is known about the metabolic and nutritional activities required by salmonellae during infection.

We tested the effect of mutations altering glutamine synthesis and/or high-affinity glutamine transport on the pathogenic properties of *S. typhimurium*. Our studies show that simultaneously preventing both glutamine synthesis and high-affinity transport attenuates *S. typhimurium* virulence. Furthermore, strains defective for both glutamine synthesis and high-affinity transport provided protective immunity to mice subsequently challenged with a wild-type *S. typhimurium* strain, demonstrating the utility of this general approach for the construction of live oral vaccines.

## MATERIALS AND METHODS

**Media.** Luria broth (LB), in both liquid medium and agar plates, was routinely supplemented with 2 mM glutamine to allow the growth of strains with reduced levels of glutamine synthetase and was supplemented with antibiotics when appropriate. Nutrient broth (NB) was used for the determination of glutamine auxotrophy (Gln<sup>-</sup> mutants cannot grow on this medium unless it is supple-

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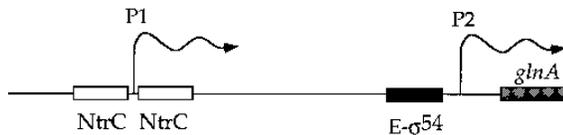


FIG. 1. Schematic representation of the *S. typhimurium glnA* promoter. The major  $\sigma^{54}$ -dependent promoter for the *glnA* gene is designated P2, and the  $\sigma^{70}$ -dependent promoter is designated P1 (arrows).  $\sigma^{54}$ -holoenzyme ( $E\text{-}\sigma^{54}$ ); core RNA polymerase associated with the *ntrA* gene product) binds in a closed complex to a site with conserved elements centered at  $-12$  and  $-24$  with respect to the start site of  $\sigma^{54}$ -dependent transcription (dark box).  $NtrC$  binds to two sites centered at  $-108$  and  $-140$  (white boxes) with respect to the  $\sigma^{54}$ -dependent start site.  $\sigma^{70}$ -dependent transcription is repressed when  $NtrC$  is bound at these two sites, and thus an *ntrA* strain is a glutamine auxotroph while an *ntrC* strain is a glutamine prototroph. A *glnAp356* mutation increases transcription from P1 and provides glutamine prototrophy to *ntrA* strains (this mutation changes a G to an A at position  $-134$  with respect to the  $\sigma^{54}$ -dependent start site, which presumably lies in the  $-10$  recognition sequence for  $\sigma^{70}$ -holoenzyme).

mented with glutamine). Morpholinepropanesulfonic acid (MOPS)-based minimal-glucose (0.4%) medium (37) with 20 mM  $\text{NH}_4\text{Cl}$  and 3 mM glutamine was used for  $\beta$ -galactosidase assays (see below). Evans blue-uranine indicator plates (37) supplemented with 2 mM glutamine were utilized to purify all constructed strains free of P22. Minimal-glucose (0.4%) agar plates with 10 mM glutamate as the sole nitrogen source were overlaid with 100  $\mu\text{l}$  of 100 mM  $\gamma$ -glutamyl hydrazide (ICN Biochemicals) to assess the growth phenotype of strains with mutations in the *glnHPQ* operon (colonies of *glnHPQ* mutants grow larger on this medium [31, 61]). LB agar, made without NaCl and supplemented with 10% sucrose, was used to select for second recombinational events during construction of chromosomal deletions or insertions with vectors containing the *sacB* gene (see below).

**Oligonucleotides and PCR.** Degenerate oligonucleotide primers based on the amino acid sequence of the *E. coli glnHPQ* genes (45) were used for PCR amplification of internal fragments from the *S. typhimurium glnH*, *glnP*, and *glnQ* genes. The primers used to amplify the *glnH* internal fragment '*glnH*' were GLNH1 (GCGGATCCCTTYGTNCCNTTYGARITTYAA) and GLNH2 (GCGGAATTCARYTCCATRTANGCRITRTRC); the underlined nucleotides represent restriction sites for *Bam*HI and *Eco*RI, respectively. The primers used to amplify the *glnP* internal fragment '*glnP*' were GLNP1 (GCGAATTCGGNACNCCNATNGTNGTNA) and GLNP2 (GCAAGCTTAGNGANGTRTCYTTNATRCT); the underlined nucleotides represent restriction sites for *Eco*RI and *Hind*III, respectively. The primers used to amplify the *glnQ* internal fragment '*glnQ*' were GLNQ1 (GCGGAATTCGGNATGGTNTTYCARCARTT) and GLNQ3 (GCGGAAGCTTACYTGNNGRRTTNCRCYCTC); the underlined nucleotides represent restriction sites for *Eco*RI and *Hind*III, respectively. In the above-described primers, N represents any nucleotide, Y represents any pyrimidine, and R represents any purine. Only one fragment was produced from strain ATCC 14028 chromosomal DNA with each pair of primers: a '*glnH*' fragment of approximately 400 bp in length, corresponding to the coding sequence for amino acids 35 to 168 of GlnH; a '*glnP*' fragment of approximately 300 bp in length, corresponding to the coding sequence for amino acids 64 to 164 of GlnP; and a '*glnQ*' fragment of approximately 430 bp in length, corresponding to the coding sequence for amino acids 80 to 222 of GlnQ. PCR was performed for 30 cycles of 45 s at 92°C, 1 min at 42°C, and 1 min 30 s at 72°C with *TaqPlus* DNA polymerase (Stratagene).

The oligonucleotides used to amplify a 461-bp internal '*argT*' fragment, based on the published sequence of the *argT* gene of *S. typhimurium* (18) and corresponding to the coding sequence for amino acids 15 to 168 of ArgT, were ARG1 (GCGGATCCGCGCGCAGCGGCGCCAGTTAC) and ARG2 (GCGAATTCGATAAGATCCTGGTTGGCATA); the underlined nucleotides represent restriction sites for *Bam*HI and *Eco*RI, respectively. The oligonucleotides used to amplify a 1.05-kbp fragment containing the chloramphenicol acetyltransferase gene from pACYC184 (53) were CAT1 (GCAATTGCGGAAGATCAC TTCGCAGAA) and CAT2 (GCCAATTGTTTGAGAAGCACACGGTTCAC); the underlined nucleotides represent restriction sites for *Mfe*I. The oligonucleotides used to amplify a 730-bp fragment containing the *glnH* promoter from ATCC 14028 (based on the derived sequence; see below) were GLNHP6 (GCTCTAGAGGAGATCTAAAGAAGTCCCGATCGCTACGCTGTTGTGTC) and GLNHP7 (GCGGTACCGGAGATCTAACGGAACGAAACGCGGTATCGTTGTC); the underlined nucleotides represent restriction sites for *Kpn*I, *Bgl*II, *Xba*I, and *Bgl*II, respectively (*Kpn*I and *Xba*I sites were introduced for other purposes). PCR was performed for 30 cycles of 45 s at 92°C, 1 min at 50°C, and 1 min 30 s at 72°C with Vent DNA polymerase (New England Biolabs).

**Plasmids.** Plasmid pJES89 (kindly provided by S. Kustu) contains the 8.9-kbp *Hind*III *S. typhimurium* chromosomal fragment containing the entire *glnA ntrBC* operon ligated into the *Hind*III site of pBR322 (59) from which the *Sal*I site had been removed. The in-frame  $\Delta$ *glnA1* was made in the following manner: the 1.8-kbp *Eco*RI fragment of pJES89 containing the *glnA* promoter and the amino-

terminal coding sequence for *glnA* (*glnA'*) was ligated into the *Eco*RI site of the temperature-sensitive suicide vector pMAK705 (15) to form pKEK3. The 4.1-kbp *Eco*RI fragment of pJES89 containing the carboxyl-terminal coding sequence for *glnA* (*glnA*) and the entire coding sequence for *ntrB* and *ntrC* was ligated into pKEK3 which had been partially digested with *Eco*RI, resulting in pKEK4, in which the *Eco*RI fragments are correctly oriented to produce an in-frame deletion within *glnA*; the resulting construct was predicted to encode a glutamine synthetase lacking amino acids 41 to 130.

To create a deletion of *ntrB* and *ntrC*, pJES89 was digested with *Sal*I and religated, removing a 2.6-kbp *Sal*I fragment which extends from within the coding sequence for the amino terminus of *ntrB* (at the codon for amino acid 23) to the *Sal*I site downstream of the coding sequence for *ntrC* (155 bp downstream of the stop codon), forming pKEK9 [ $\Delta$ (*ntrB-ntrC*)1]. The 1.6-kbp partial *Eco*RI-*Hind*III fragment of pKEK9 containing the carboxyl-terminal portion of *glnA* (starting at the codon for amino acid 131) and  $\Delta$ (*ntrB-ntrC*)1 was ligated into pBR322 (59) that had been digested with *Eco*RI and *Hind*III, forming pKEK10. pKEK9 was also digested with *Sal*I, partially digested with *Bgl*II, made blunt-ended with the Klenow fragment of DNA polymerase, and then religated to form pKEK34, from which all coding sequence for *glnA*, *ntrB*, and *ntrC* has been removed [ $\Delta$ (*glnA-ntrC*)1]; the deletion begins at the *Bgl*II site within the *glnA* promoter (centered at +28 with respect to the start site of transcription from the  $\sigma^{54}$ -dependent promoter) and extends to the *Sal*I site downstream of *ntrC*. The *Bgl*II-*Hind*III fragment from pKEK34 containing  $\Delta$ (*glnA-ntrC*)1 was ligated into pMAK705 (15) digested with *Bam*HI and *Hind*III, forming pKEK55.

To make plasmids for insertion mutations, the PCR-amplified fragments of *S. typhimurium* '*glnH*' and '*argT*' (see above) were digested with *Bam*HI and *Eco*RI and ligated into pBR322 (59) digested with *Bam*HI and *Eco*RI, forming pKEK11 ('*glnH*') and pKEK56 ('*argT*'), respectively; the PCR-amplified fragments of *S. typhimurium* '*glnP*' and '*glnQ*' (see above) were digested with *Eco*RI and *Hind*III and ligated into pBR322 (59) digested with *Eco*RI and *Hind*III, forming pKEK37 ('*glnP*') and pKEK12 ('*glnQ*'), respectively. pKEK11 and pKEK56 were digested with *Bam*HI, made blunt-ended with the Klenow fragment, digested with *Eco*RI, and then ligated into the suicide vector pGP704 (42) that had been digested with *Eco*RI and *Eco*RV, resulting in pKEK13 and pKEK57. pKEK12 was digested with *Hind*III, made blunt-ended with the Klenow fragment, digested with *Eco*RI, and then ligated into pGP704 (42) that had been digested with *Eco*RI and *Eco*RV, forming pKEK14.

The  $\Delta$ (*glnH-P*):*Cm*' mutation was made as follows: the *Bam*HI (made blunt-ended with Klenow fragment)-*Eco*RI fragment from pKEK11 (see above) was first ligated into pKEK37 that had been digested with *Aat*II, made blunt-ended with mung bean nuclease, and then digested with *Eco*RI, forming pKEK58, which contains '*glnH*' and '*glnP*' in the same orientation to form  $\Delta$ (*glnH-P*)1, which removes amino acids 169 to 248 from the coding sequence of GlnH, the *glnHQ* intergenic region, and amino acids 1 to 63 from the coding sequence of GlnP. The PCR-amplified chloramphenicol resistance gene from pACYC184 (53) (see above) was digested with *Mfe*I and ligated into pKEK58 that had been digested with *Eco*RI, forming pKEK59 [ $\Delta$ (*glnH-P*):*Cm*']. pKEK59 was used as a template in PCR amplification with GLNH1 and GLNP2 (see above) and ligated into the suicide vector pCVD442 (8) digested with *Sma*I, forming pKEK60.

For sequencing the *glnHPQ* operon, chromosomal DNA from strain KK110 (see below) was purified, digested to completion with *Hind*III, and then ligated into pWSK30 (58), with selection for a plasmid conferring chloramphenicol resistance. The resulting plasmid, pKEK61, contains  $\Delta$ (*glnH-P*):*Cm*' within an approximately 15-kbp chromosomal fragment.

To make a chromosomal *glnHp-lacZ* fusion, the PCR-amplified *glnHp67* fragment (see above) was digested with *Bgl*II and ligated into pRS551 (56) digested with *Bam*HI. A plasmid containing the correct *glnHp-lacZ* fusion was named pKEK69.

**Bacterial strains.** *E. coli* DH5 $\alpha$  (16) was used for all cloning manipulations unless the vector being used was a derivative of pGP704 (42) or pCVD442 (8), which contain the R6K origin of replication and therefore require the product of the *pir* gene for replication, in which case *E. coli* DH5 $\alpha$ pir or SM10 $\lambda$ pir (42) was used. For construction of the *glnHp-lacZ* chromosomal fusion, *E. coli* TE2680 and TE1335 (10) were used in intermediate steps (see below).

*S. typhimurium* strains used in this study are listed in Table 1. All *S. typhimurium* strains used are isogenic with strain ATCC 14028, referred to as the wild type. Mutant strains were constructed utilizing the high-transducing phage P22 HT *int* (55), and their construction is outlined in Table 1, the paternal donor upon which the P22 lysate was made being listed first, followed by the recipient. P22 phage lysate from SK811 (*zig214::Tn10 hisF645* [29]) or SK2899 (*zig::Kan<sup>r</sup> hisF645* [27]) was used to transduce strain SK35 [ $\Delta$ (*glnA-ntrC*)60 *hisF645*] (29, 39) to tetracycline or kanamycin resistance, respectively, while retaining glutamine auxotrophy, thus linking the deletion to an antibiotic resistance marker; the resulting strains were used as donors to transduce ATCC 14028 to tetracycline or kanamycin resistance and glutamine auxotrophy, forming strains KK4 [ $\Delta$ (*glnA-ntrC*)60 *zig214::Tn10*] and KK38 [ $\Delta$ (*glnA-ntrC*)60 *zig::Kan<sup>r</sup>*], respectively. The *zig214::Tn10* insertion has been precisely mapped to a location lying in the promoter region of *hemN* (21), which lies downstream of *ntrC*; because strains with this insertion maintain a *HemN*<sup>+</sup> phenotype, strain KK14 (*zig214::Tn10*) was used as the wild type in competition assays (see below).

To make a strain with the *glnAp356* mutation, P22 phage lysate from strain

TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
14028	Wild type	American Type Culture Collection
CS015	<i>phoP102::Tn10d-Cam</i>	41
KK1	<i>ntxA209::Tn10</i>	P22.SK284 × 14028
KK2	<i>ntxC352::Tn10</i>	P22.SK835 × 14028
KK4	$\Delta(glnA-ntxC)60$ <i>zig214::Tn10</i>	This study
KK6	<i>glnAp356</i>	P22.SK840 × KK4
KK8	<i>glnAp356 ntrA209::Tn10</i>	P22.SK284 × KK6
KK10	<i>ntxB137::Tn10</i>	P22.SK398 × 14028
KK13	<i>invA::aphT</i>	P22.SB147 × 14028
KK14	<i>zig214::Tn10</i>	P22.SK811 × 14028
KK16	<i>phoP102::Tn10d-Cam</i>	P22.CS015 × 14028
KK26	$\Delta himD::Cm^r$	P22.SK2842 × 14028
KK27	$\Delta glnA1$	This study
KK30	$\Delta glnA1$ <i>ntrA209::Tn10</i>	P22.SK284 × KK27
KK32	$\Delta glnA1$ $\Delta himD::Cm^r$	P22.KK26 × KK27
KK34	$\Delta glnA1$ <i>ntxC352::Tn10</i>	P22.SK835 × KK27
KK38	$\Delta(glnA-ntxC)60$ <i>zig::Kan^r</i>	This study
KK44	<i>glnH1::pGP704</i>	This study
KK45	$\Delta glnA1$ <i>glnH1::pGP704</i>	P22.KK44 × KK27
KK49	<i>glnQ1::pGP704</i>	This study
KK50	$\Delta glnA1$ <i>glnQ1::pGP704</i>	P22.KK49 × KK27
KK55	<i>ntrA209::Tn10 putPA1303[Kan^r-glnAp-lacZYA]</i>	P22.SK3041 × KK1
KK56	<i>ntxC352::Tn10 putPA1303[Kan^r-glnAp-lacZYA]</i>	P22.SK3041 × KK2
KK57	$\Delta(glnA-ntxC)60$ <i>putPA1303[Kan^r-glnAp-lacZYA]</i>	P22.SK3041 × KK4
KK58	$\Delta glnA1$ <i>putPA1303[Kan^r-glnAp-lacZYA]</i>	P22.SK3041 × KK27
KK59	$\Delta glnA1$ <i>ntrA209::Tn10 putPA1303[Kan^r-glnAp-lacZYA]</i>	P22.SK3041 × KK30
KK60	<i>glnH1::pGP704 putPA1303[Kan^r-glnAp-lacZYA]</i>	P22.SK3041 × KK44
KK61	$\Delta glnA1$ <i>glnH1::pGP704 putPA1303[Kan^r-glnAp-lacZYA]</i>	P22.SK3041 × KK45
KK62	<i>putPA1303[Kan^r-glnAp-lacZYA]</i>	P22.SK3041 × 14028
KK76	$\Delta glnA1$ <i>hisJ8908::Tn10</i>	P22.TA3178 × KK27
KK79	$\Delta(ntrB-C)1$ $\Delta hisF645$	This study
KK80	$\Delta(ntrB-C)1$	P22.KK79 × KK38
KK82	<i>argT1::pGP704</i>	This study
KK83	$\Delta glnA1$ <i>argT1::pGP704</i>	P22.KK82 × KK27
KK85	$\Delta(glnA-ntxC)1$	This study
KK110	$\Delta(glnH-P)1::Cm^r$ $\Delta glnA1$	P22.KK111 × KK27
KK111	$\Delta(glnH-P)1::Cm^r$	This study
KK116	<i>putPA1303[Kan^r-glnHp-lacZYA]</i>	This study
KK117	<i>putPA1303[Kan^r-glnHp-lacZYA]</i>	P22.KK116 × 14028
KK118	<i>ntrA209::Tn10 putPA1303[Kan^r-glnHp-lacZYA]</i>	P22.KK116 × KK1
KK119	<i>ntxC352::Tn10 putPA1303[Kan^r-glnHp-lacZYA]</i>	P22.KK116 × KK2
KK120	$\Delta(glnA-ntxC)60$ <i>putPA1303[Kan^r-glnHp-lacZYA]</i>	P22.KK116 × KK4
KK121	$\Delta glnA1$ <i>putPA1303[Kan^r-glnHp-lacZYA]</i>	P22.KK116 × KK27
KK122	$\Delta glnA1$ <i>ntrA209::Tn10 putPA1303[Kan^r-glnHp-lacZYA]</i>	P22.KK116 × KK30
KK123	<i>glnH1::pGP704 putPA1303[Kan^r-glnHp-lacZYA]</i>	P22.KK116 × KK44
KK124	$\Delta glnA1$ <i>glnH1::pGP704 putPA1303[Kan^r-glnHp-lacZYA]</i>	P22.KK116 × KK45
KK125	$\Delta himD::Cm^r$ <i>putPA1303[Kan^r-glnHp-lacZYA]</i>	P22.KK116 × KK26
KK126	$\Delta glnA1$ $\Delta himD::Cm^r$ <i>putPA1303[Kan^r-glnHp-lacZYA]</i>	P22.KK116 × KK32
KK127	$\Delta himD::Cm^r$ <i>putPA1303[Kan^r-glnAp-lacZYA]</i>	P22.SK3041 × KK26
KK128	$\Delta glnA1$ $\Delta himD::Cm^r$ ; <i>putPA1303[Kan^r-glnAp-lacZYA]</i>	P22.SK3041 × KK32
KK129	<i>putPA1303[Kan^r-lacUV5p-lacUV5p-lacZYA]</i>	P22.SK3082 × 14028
KK130	<i>ntrA209::Tn10 putPA1303[Kan^r-lacUV5p-lacZYA]</i>	P22.SK3082 × KK1
KK131	<i>ntxC352::Tn10 putPA1303[Kan^r-lacUV5p-lacZYA]</i>	P22.SK3082 × KK2
KK132	$\Delta(glnA-ntxC)60$ <i>putPA1303[Kan^r-lacUV5p-lacZYA]</i>	P22.SK3082 × KK4
KK133	$\Delta glnA1$ <i>putPA1303[Kan^r-lacUV5p-lacZYA]</i>	P22.SK3082 × KK27
KK134	$\Delta glnA1$ <i>ntrA209::Tn10 putPA1303[Kan^r-lacUV5p-lacZYA]</i>	P22.SK3082 × KK30
KK135	<i>glnH1::pGP704 putPA1303[Kan^r-lacUV5p-lacZYA]</i>	P22.SK3082 × KK44
KK136	$\Delta glnA1$ <i>glnH1::pGP704 putPA1303[Kan^r-lacUV5p-lacZYA]</i>	P22.SK3082 × KK45
KK137	$\Delta himD::Cm^r$ <i>putPA1303[Kan^r-lacUV5p-lacZYA]</i>	P22.SK3082 × KK26
KK138	$\Delta glnA1$ $\Delta himD::Cm^r$ <i>putPA1303[Kan^r-lacUV5p-lacZYA]</i>	P22.SK3082 × KK32
KK139	<i>putPA1303[Kan^r-lacZYA]</i>	This study
KK140	<i>putPA1303[Kan^r-lacZYA]</i>	P22.KK139 × 14028
SB147	<i>invA::aphT</i>	13
SK35	$\Delta(glnA-ntxC)60$ <i>hisF645</i>	39
SK284	<i>ntrA209::Tn10 hisF645</i>	29
SK398	<i>ntxB137::Tn10 hisF645</i>	29
SK811	<i>zig214::Tn10 hisF645</i>	29
SK835	<i>ntxC352::Tn10 hisF645</i>	29
SK840	<i>glnAp356 hisF645</i>	28
SK2842	$\Delta himD::Cm^r$ $\Delta(ntrB-C)::Tet^r$ <i>hisF645</i>	27
SK2899	<i>zig::Kan^r hisF645</i>	27
SK3041	<i>putPA1303[Kan^r-glnAp-lacZYA]</i>	22
SK3082	<i>putPA1303[Kan^r-lacUV5p-lacZYA]</i>	22
SL4213	<i>galE496 metA22 metE55 rpsL120 xyl-404 (Fels2)<sup>-</sup> H1-b nml<sup>-</sup> H2-enx (ilv?)<sup>-</sup> hsdL6 hsdSA29</i>	11
SL4213 $\lambda$ pir	<i>galE496 metA22 metE55 rpsL120 xyl-404 (Fels2)<sup>-</sup> H1-b nml<sup>-</sup> H2-enx (ilv?)<sup>-</sup> hsdL6 hsdSA29/F'100-12<math>\lambda</math>pir</i>	35
TA3178	<i>hisJ8908::Tn10</i>	30

SK840 (*glnAp356 hisF645* [38]) was used to transduce KK4 [ $\Delta$ (*glnA-ntrC*)60 *zig214::Tn10*] to glutamine prototrophy on NB agar; a tetracycline-sensitive transductant (KK6), which was shown to contain the *glnAp356* mutation by a transductional backcross, was used as a recipient in a cross with phage lysate from SK284 (*ntrA209::Tn10 hisF645* [29]) to form strain KK8 (*glnAp356 ntrA209::Tn10*); the *glnAp356* mutation is a suppressor of glutamine auxotrophy in an *ntrA* background).

Strain KK27 ( $\Delta$ *glnA1*) was made by first transforming ATCC 14028 with pKEK4 (see above) and selecting for a Cm<sup>r</sup> colony at 30°C, the permissive temperature for replication of this plasmid. This 14028 strain harboring pKEK4 was grown in LB with 2 mM glutamine to mid-log phase at 30°C, then plated on LB supplemented with 2 mM glutamine and 20 µg of chloramphenicol per ml and allowed to grow overnight at 42°C, at which temperature the plasmid cannot replicate, thus selecting for cointegrants. Several Cm<sup>r</sup> colonies were restreaked, allowed to grow on the same medium at 42°C, inoculated into LB plus 2 mM glutamine, and grown for two rounds at the replication permissive temperature of 30°C to saturation. This culture was diluted and plated on LB plus 2 mM glutamine at 30°C; colonies were then patched onto NB and LB plus 2 mM glutamine and 20 µg of chloramphenicol per ml to identify a Gln<sup>-</sup> Cm<sup>r</sup> strain, indicating that a second recombinational event had occurred to create a chromosomal deletion. Strain KK27 was shown to contain the correct chromosomal  $\Delta$ *glnA1* by PCR and sequence analysis.

Strains KK44, KK49, and KK82 were constructed by first electroporating plasmids pKEK13, pKEK14, and pKEK57, respectively, into SL4213F<sup>λ</sup>*pir* (35) to obtain protective methylation; then these plasmids were electroporated into ATCC 14028 and plated on LB plus 2 mM glutamine and 100 µg of ampicillin per ml at 37°C. These plasmids contain internal gene fragments in a vector which requires the *pir* gene product for replication, so the resulting strains have insertions caused by the coinTEGRATION of pKEK13 (*glnH1::pGP704* [Amp<sup>r</sup>]), pKEK14 (*glnQ1::pGP704* [Amp<sup>r</sup>]), and pKEK57 (*argT1::pGP704* [Amp<sup>r</sup>]); the mutations were moved by P22-mediated transduction into ATCC 14028 to form strains KK44, KK49, and KK82, respectively. Strains KK44 and KK49 were confirmed to have insertions in the *glnHPQ* operon by growth phenotype (see above), and KK82 was shown to have an insertion in *argT* by transductional linkage; insertions were also confirmed by Southern blot analysis.

Strain KK85, which contains a complete deletion of all coding sequence of the *glnA ntrBC* operon [ $\Delta$ (*glnA-ntrC*)1], was constructed in several steps. First, pKEK10 (see above) was electroporated into SK35 [ $\Delta$ (*glnA-ntrC*)60 *hisF645*] (29, 39), and colonies were selected by growth on NB agar, on which only glutamine prototrophs can grow; SK35 contains a partial deletion that extends from the C-terminal coding region of *glnA* to the amino-terminal coding region of *ntrC*, and pKEK10 lacks the amino-terminal coding region of *glnA*. This selection resulted in the formation of KK79 by a double-crossover event [ $\Delta$ (*ntrB-C*)1 *hisF645*]. The  $\Delta$ (*ntrB-C*)1 mutation was moved into the ATCC 14028 background by P22-mediated transduction into KK38 with selection for Gln<sup>+</sup> on NB agar; a Kan<sup>r</sup> transductant was chosen, indicating that *zig::Kan<sup>r</sup>* had been replaced during the transduction, to give KK80 [ $\Delta$ (*ntrB-C*)1]. pKEK55 was then electroporated into KK80, with selection for Cm<sup>r</sup> at 30°C and subsequent selection at 42°C followed by growth at 30°C as outlined above for KK27, resulting in strain KK85 [ $\Delta$ (*glnA-ntrC*)1]. The correct chromosomal deletion was confirmed by both PCR and Southern blot analysis.

Strains containing the  $\Delta$ (*glnH-P*):Cm<sup>r</sup> mutation were made by performing the following steps. pKEK60 was mated into KK14 (*zig214::Tn10*) from *E. coli* SM10*pir* (42) by streaking the two strains together on LB plus 2 mM glutamine, incubating them overnight, and then streaking for single colonies on LB supplemented with 2 mM glutamine, 100 µg of ampicillin per ml, and 12 µg of tetracycline per ml. Single colonies were grown overnight in LB plus 2 mM glutamine at 37°C and then diluted 1:1,000 in phosphate-buffered saline (PBS), and 100 µl was plated on LB plus 2 mM glutamine and 10% sucrose and grown overnight at 30°C. The integrated plasmid contains the *sacB* gene (8), the expression of which is lethal on this medium; thus, there is selection for a second recombinational event. Sucrose-resistant colonies were tested for antibiotic resistance; a Cm<sup>r</sup> Amp<sup>r</sup> strain was used as the donor for P22-mediated transduction into ATCC 14028 and KK27, forming strains KK111 and KK110, respectively. Confirmation of correct chromosomal integration into the *glnHPQ* operon was obtained by sequencing the flanking DNA (see below), and the nature of the mutation was confirmed by phenotypic characterization (see above).

The creation of the *glnHp-lacZ* chromosomal fusion cassette inserted into the *putPA* locus is briefly outlined here; the technique is more thoroughly explained elsewhere (10, 22). pKEK69 was linearized by cleavage with *Pst*I and electroporated into *E. coli* TE2680 (10). A Kan<sup>r</sup> Cm<sup>r</sup> Lac<sup>+</sup> strain was chosen, indicating that the *glnHp-lacZ* fusion had been correctly integrated into the chromosome. This *E. coli* intermediate strain was then mated with *E. coli* TE1335 (which contains lysogenized P22 [9]) to form a P22 lysate by zygotic induction, which was then used to transduce *S. typhimurium* SL4213 (r<sup>-</sup> m<sup>+</sup> [11]) to Kan<sup>r</sup>, forming KK116. This strain thus contains the insertion *putPA1303::[Kan<sup>r</sup>-glnHp-lacZYA]*. As a control, plasmid without insert, pRS551 (56), was inserted into the *S. typhimurium* SL4213 chromosome in the same manner to form KK139. The *glnHp-lacZ* fusion from KK116 and the promoterless insertion in KK139, as well as the *glnAp-lacZ* fusion from SK3041 and *lacUV5p-lacZ* fusion from SK3082 (22), were transferred by P22-mediated transduction to all other *S. typhimurium* strains listed in Table 1.

TABLE 2. Single mutations in genes involved with glutamine synthesis or high-affinity transport allow full virulence

Strain	Genotype	i.p. LD <sub>50</sub>	Oral LD <sub>50</sub>	Competitive index (i.p.) <sup>a</sup>
14028	Wild type	<10	<5 × 10 <sup>6</sup>	0.807 <sup>b</sup>
KK1	<i>ntrA209::Tn10</i>	<10	<5 × 10 <sup>6</sup>	0.0087
KK2	<i>ntrC352::Tn10</i>	<10		1.27
KK10	<i>ntrB137::Tn10</i>	<10		1.021
KK27	$\Delta$ <i>glnA1</i>	<10		0.071
KK44	<i>glnH1::pGP704</i>	<10		1.869
KK49	<i>glnQ1::pGP704</i>	<10		0.666
KK8	<i>ntrA209::Tn10 glnAp356</i>	<10		0.368
KK111	$\Delta$ ( <i>glnH-P</i> ):Cm <sup>r</sup>	<10		0.866

<sup>a</sup> The experiment was performed according to the procedure outlined in Materials and Methods. The competitive index is reported as the ratio of output bacteria (mutant/wild type) recovered from the spleen divided by the ratio of input bacteria (mutant/wild type) inoculated i.p. Numbers are the averages of values for two mice per inoculum. The closer the mutant resembles the wild type, the closer the index is to 1.

<sup>b</sup> Strain KK14 (*zig214::Tn10*) was used as the wild type (see Materials and Methods) in competition with 14028; input and output were thus scored for Tet<sup>r</sup>.

**Sequencing.** Cycle dideoxynucleotide sequencing was carried out with an ABI sequencing kit and an ABI model 373AStretch sequencer. The complete nucleotide sequence of the *S. typhimurium glnHPQ* operon was determined from both strands of pKEK61 by using specific oligonucleotide primers; the portion of '*glnH-glnQ*' sequence not contained in this plasmid was determined from a PCR product derived from amplification of ATCC 14028 chromosomal DNA with specific primers, utilizing Vent DNA polymerase (New England BioLabs) to virtually eliminate any mispriming.

**β-Galactosidase assays.** Strains were grown overnight in MOPS (0.4% glucose) minimal medium (37) with 20 mM NH<sub>4</sub>Cl and 3 mM glutamine, then reinoculated into the same medium and grown at 37°C. Samples were assayed at an optical density at 600 nm of approximately 0.2 to 0.4, permeabilized with chloroform and sodium dodecyl sulfate, and assayed for β-galactosidase activity according to the method of Miller (40).

**Tissue culture assays.** J774 cells were grown in RPMI 1640 buffered with 25 mM HEPES (Cellgro/Mediatech) and supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO/BRL), penicillin-streptomycin (50 U/ml and 50 µg/ml, respectively; GIBCO/BRL), and 2 mM glutamine. HEP-2 cells were grown without antibiotics in minimal essential medium (Cellgro/Mediatech) supplemented with 5% FBS and 2 mM glutamine. Wells of 24-well (16-mm) microtiter culture dishes were seeded with 5 × 10<sup>5</sup> J774 cells or HEP-2 cells for macrophage survival and invasion assays, respectively, and incubated overnight at 37°C in 5% CO<sub>2</sub>. The macrophage survival assay (see Fig. 3) was carried out essentially as described previously (34), with the following alterations. J774 cells were washed three times with PBS and overlaid with RPMI 1640 containing 5% FBS, with or without 2 mM glutamine (no antibiotics). Bacteria were grown aerobically to stationary phase, washed once with PBS, diluted in PBS containing 1% mouse immune serum (from mice that survived inoculation with strain KK4 and subsequent challenge with 14028; opsonization of *S. typhimurium* enhances uptake into macrophages), and incubated for 30 min at 37°C. Bacteria (5 × 10<sup>6</sup>) were added to each well; the culture dish was centrifuged for 10 min at 1,000 rpm and then incubated for 30 min at 37°C in 5% CO<sub>2</sub>. Wells were washed three times with PBS, overlaid with RPMI 1640 containing 5% FBS plus 5 µg of gentamicin per ml (an empirically derived minimum bactericidal concentration) with or without 2 mM glutamine, and incubated at 37°C in 5% CO<sub>2</sub>. At various time points, the wells to be assayed were washed three times with PBS, lysed with 500 µl of 0.5% sodium desoxycholate, and then rinsed with 500 µl of PBS; the lysate and rinse were combined, diluted, and plated on LB agar supplemented with 2 mM glutamine to determine CFUs. For the HEP-2 invasion assay, bacteria were grown under oxygen-limited conditions as previously described (32), and the invasion assay was carried out as previously described (2).

**Mouse virulence assays.** Female 6- to 8-week-old BALB/c mice (Charles River Breeding Facility) were used in all studies. *S. typhimurium* strains were grown overnight at 37°C to stationary phase in LB plus 2 mM glutamine, washed once, diluted to an appropriate concentration in PBS (for intraperitoneal inoculations) or 0.1 M NaHCO<sub>3</sub> (for peroral inoculations), and administered to animals in 100-µl inocula; control animals received either PBS or NaHCO<sub>3</sub> alone. Inocula were plated on LB plus 2 mM glutamine to determine CFUs. For 50% lethal dose (LD<sub>50</sub>) studies, groups of five mice were inoculated per dilution; survival was measured for 4 weeks postinoculation. For vaccine studies, mice that survived 4 weeks past administration of the initial inoculum were then challenged with ATCC 14028 (wild type) administered either intraperitoneally (i.p.) or perorally. For competition assays, strains were mixed to give inocula with a 1:1 or 100:1 ratio of mutant to wild type (the actual numbers of organisms inoculated

TABLE 3. Combination of  $\Delta glnA$  with mutations in  $ntrA$ ,  $ntrC$ ,  $glnH$ , or  $glnQ$  attenuates virulence

Strain	Genotype	i.p. LD <sub>50</sub>	Oral LD <sub>50</sub>	Competitive index (i.p.) <sup>a</sup>
14028	Wild type	<10	<5 × 10 <sup>6</sup>	0.807 <sup>b</sup>
KK4	$\Delta(glnA-ntrC)60$ <i>zig214::Tn10</i>	>1.5 × 10 <sup>5</sup>	>1.8 × 10 <sup>9</sup>	<0.0001
KK85	$\Delta(glnA-ntrC)1$	>1.5 × 10 <sup>5</sup>		<0.0001
KK30	$\Delta glnA1$ <i>ntrA209::Tn10</i>	>1.5 × 10 <sup>5</sup>		<0.0001
KK34	$\Delta glnA1$ <i>ntrC352::Tn10</i>	>1.5 × 10 <sup>5</sup>		<0.0001
KK45	$\Delta glnA1$ <i>glnH1::pGP704</i>	>1.5 × 10 <sup>5</sup>	>1.5 × 10 <sup>9</sup>	<0.0001
KK50	$\Delta glnA1$ <i>glnQ1::pGP704</i>	>1.5 × 10 <sup>5</sup>		<0.0001
KK110	$\Delta glnA1$ $\Delta(glnH-P)1::Cm^r$	>1.5 × 10 <sup>5</sup>		<0.0001
KK16	<i>phoP102::Tn10dCam</i>	>1.5 × 10 <sup>5</sup>		<0.0001
KK26	$\Delta himD::Cm^r$	~1.5 × 10 <sup>5</sup>		<0.0001
KK32	$\Delta glnA1$ $\Delta himD::Cm^r$	>1.5 × 10 <sup>5</sup>		<0.0001
KK76	$\Delta glnA1$ <i>hisJ8908::Tn10</i>	<10		ND <sup>c</sup>
KK83	$\Delta glnA1$ <i>argT1::pGP704</i>	<10		ND

<sup>a</sup> The experiment was performed according to the procedure outlined in Materials and Methods. The competitive index is reported as the ratio of output bacteria (mutant/wild type) recovered from the spleen divided by the ratio of input bacteria (mutant/wild type) inoculated i.p. Numbers are the averages of the values for two mice per inoculum. The closer the mutant resembles the wild type, the closer the index is to 1.

<sup>b</sup> This is the same experiment as reported in Table 2. Strain KK14 (*zig214::Tn10*) was used as the wild type (see Materials and Methods) in competition with 14028; input and output were thus scored for Tet<sup>r</sup>.

<sup>c</sup> ND, not determined.

i.p. were close to 500:500 or 5,000:50, respectively), and the input ratio was determined by plating and scoring a minimum of 200 colonies for either antibiotic resistance or glutamine auxotrophy, whichever was relevant. Two mice were inoculated per mixture; when mice were moribund (typically 4 to 5 days), they were sacrificed, their spleens were removed, and the output ratios were determined in tissue homogenates by plating on appropriate media.

**Nucleotide sequence accession number.** The complete sequence of the *S. typhimurium glnHPQ* operon has been deposited in GenBank under accession number U73111.

## RESULTS

**Single mutations in genes involved with glutamine synthesis do not affect the virulence of *S. typhimurium*.** *S. typhimurium* strains with single mutations in either *glnA* (glutamine synthetase) or one of the genes that regulate *glnA* transcription (*ntrA*, *ntrB*, or *ntrC*) were tested for virulence properties in BALB/c mice. We utilized both a competition assay, to determine a mutant's fitness relative to that of the wild-type strain, and an LD<sub>50</sub> assay to determine the effect of these mutations on *S. typhimurium* virulence (Table 2). An *ntrB* or *ntrC* strain (KK10 or KK2, respectively) showed no significant defect in virulence either in a competition assay or in an i.p. LD<sub>50</sub> assay (the *Tn10* insertion in *ntrB* is polar on *ntrC*, and these strains are thus *ntrBC*). A  $\Delta glnA$  strain (KK27) was approximately 10-fold less competitive and an *ntrA* strain (KK1) was approximately 100-fold less competitive than an isogenic wild-type strain, and yet both these strains had i.p. LD<sub>50</sub>s identical to that of wild-type. The average number of days until death at an i.p. inoculum of ~10 bacteria was also noticeably longer in mice infected with the *ntrA* strain than in mice infected with the wild-type strain (10.6 vs. 6.2) (data not shown). The *ntrA* strain had an oral LD<sub>50</sub> comparable to that of the wild-type strain. The competitive defect in an *ntrA* strain was significantly overcome by the introduction of *glnAp356*, a suppressing mutation which makes an *ntrA* strain a glutamine prototroph (strain KK8 [Table 1]) (this mutation increases transcription from the  $\sigma^{70}$ -dependent *glnA* promoter P1 [Fig. 1] [38]). These results indicate that the competitive defect in an *ntrA* strain is primar-

ily due to low levels of *glnA* transcription and not to the absence of some other gene transcribed by  $\sigma^{54}$ -holoenzyme.

**Combination of  $\Delta glnA$  with *ntrA* or *ntrC* attenuates *S. typhimurium* virulence.** We combined the in-frame *glnA* deletion mutation with mutations in the regulatory gene *ntrA* or *ntrC* and tested these strains for virulence properties in BALB/c mice.  $\Delta glnA$  *ntrA* (KK30) and  $\Delta glnA$  *ntrC* (KK34) strains were more than 10<sup>4</sup>-fold less competitive than an isogenic wild-type strain and had i.p. LD<sub>50</sub> values at least 4 orders of magnitude higher than that of the wild-type strain (Table 3). Moreover, partial and complete deletions of the *glnA ntrBC* operon [ $\Delta(glnA-ntrC)60$  and  $\Delta(glnA-ntrC)1$ ; KK4 and KK85, respectively] were also more than 10<sup>4</sup>-fold less competitive and had i.p. LD<sub>50</sub> values at least 4 orders of magnitude higher than that of the wild-type strain (Table 3). KK4 also had an oral LD<sub>50</sub> at least 3 orders of magnitude higher than that of the wild-type strain. These levels of attenuation are similar to those seen in a strain carrying a mutation in the virulence regulatory gene *phoP* (KK16) (Table 3).

**The high-affinity glutamine transport operon *glnHPQ* is transcribed by the *ntr* system.** The data presented above indicate that in a  $\Delta glnA$  strain, *ntrA* and *ntrC* are required to transcribe a gene(s) for glutamine acquisition to allow full virulence. We suspected that the high-affinity glutamine transport operon *glnHPQ* might fulfill this role because the *glnH* gene product has been shown to be coregulated along with glutamine synthetase by the *ntr* system in *S. typhimurium* (31) and because the *glnH* promoter from *E. coli* is transcribed by  $\sigma^{54}$  (NtrA) and NtrC in a purified in vitro system (6).

To investigate this possibility, we first cloned and sequenced the entire *S. typhimurium glnHPQ* operon: the predicted gene

TABLE 4. Transcription of the *glnH* promoter is  $\sigma^{54}$  and NtrC dependent<sup>a</sup>

Genotype	$\beta$ -Galactosidase activity in strain with mutation		
	<i>glnHp-lacZ</i> <sup>b</sup>	<i>glnAp-lacZ</i> <sup>c</sup>	<i>lacUV5p-lacZ</i> <sup>d</sup>
Wild type	2978	2401	2789
<i>ntrA::Tn10</i>	231	71	2970
<i>ntrC::Tn10</i>	528	290	3990
$\Delta glnA$	2871 <sup>e</sup>	4883	2632
$\Delta(glnA-ntrC)$	369	170	2913
$\Delta glnA$ <i>ntrA::Tn10</i>	185	117	2985
<i>glnH</i>	2367	3758	2130
$\Delta glnA$ <i>glnH</i>	4085	7171	3722
$\Delta himD$	1462	705 <sup>f</sup>	4173
$\Delta glnA$ $\Delta himD$	1362	4242	1793

<sup>a</sup> Assays were performed according to procedures outlined in Materials and Methods. Strains were grown in MOPS (0.4%) glucose minimal medium with 20 mM NH<sub>4</sub>Cl and 3 mM glutamine; cultures were assayed at optical density at 600 nm of ~0.2 to 0.4. Results are expressed in Miller units (40). Strain KK140 (*putPA::lacZ*) grown in this medium has 8 Miller units of activity, which can be considered background activity.

<sup>b</sup> The actual strains used (Table 1) were KK117, KK118, KK119, KK121, KK120, KK122, KK123, KK124, KK125, and KK126.

<sup>c</sup> The actual strains used (Table 1) were KK62, KK55, KK56, KK58, KKK57, KK59, KK60, KK61, KK127, and KK128.

<sup>d</sup> The actual strains used (Table 1) were KK129, KK130, KK131, KK133, KK132, KK134, KK135, KK136, KK137, and KK138.

<sup>e</sup> Krajewska-Gryniewicz and Kustu reported 10-fold increases in glutamine-binding protein (GlnH) over that of wild-type in *glnA* strains (29); we suspect that our failure to see a 10-fold increase in this assay is due to one of the following: (i) differences in strain background, (ii) differences in assay conditions (e.g., log versus stationary phase, media used), or (iii) difference in measuring *glnH* transcription versus GlnH antigen.

<sup>f</sup> We are unable to explain this reproducible decrease in *glnA* transcription in a *himD* background; the effect of IHF on *glnA* transcription has never been studied.



TABLE 5. Virulence and protective efficacy of *Salmonella* strains

Strain	Immunizing dose	Initial survivors/total	Survivors/total with wild-type challenge of:	
			$1.5 \times 10^5$ cells (i.p.)	$10^9$ cells (oral)
None		10/10	0/5	0/5
KK4 [ $\Delta(glnA-ntrC)60$ ]	10	5/5	5/5	
	$1.6 \times 10^2$	5/5	5/5	
	$1.6 \times 10^3$	5/5	5/5	
	$7 \times 10^3$	4/4	4/4	
	$1.6 \times 10^4$	5/5	5/5	
	$1.6 \times 10^5$	10/10	5/5	5/5
	$1.8 \times 10^9$ (oral)	9/10	4/4	5/5
KK85 [ $\Delta(glnA-ntrC)1$ ]	$1.5 \times 10^5$	5/5	5/5	
KK30 ( $\Delta glnA1 ntrA209::Tn10$ )	10	5/5	5/5	
	$1.5 \times 10^5$	4/4	4/4	
KK34 ( $\Delta glnA1 ntrC352::Tn10$ )	10	4/5	2/4	
	$1.5 \times 10^5$	4/5	4/4	
KK45 ( $\Delta glnA1 glnH1::pGP704$ )	10	10/10	0/5	
	$1.5 \times 10^2$	5/5	4/5	
	$1.5 \times 10^3$	5/5	5/5	
	$1.5 \times 10^4$	5/5	5/5	
	$1.5 \times 10^5$	10/10	5/5	5/5
	$1.5 \times 10^9$ (oral)	10/10	5/5	5/5
KK50 ( $\Delta glnA1 glnQ1::pGP704$ )	10	5/5	1/5	
	$1.5 \times 10^5$	4/5	4/4	
KK110 [ $\Delta glnA1 \Delta(glnH-P)1::Cm^r$ ]	$1.5 \times 10^5$	5/5	5/5	
KK26 ( $\Delta himD::Cm^r$ )	10	4/5	0/4	
	$1.5 \times 10^5$	3/5	2/3	
KK32 ( $\Delta glnA1 \Delta himD::Cm^r$ )	$1.5 \times 10^5$	5/5	0/5	
KK16 ( $\Delta phoP102::Tn10dCam$ )	$1.5 \times 10^5$	5/5	2/5	

strain, but a  $\Delta(glnA-ntrC)$  strain (KK4) was defective for intracellular survival, similar to the defect of a strain carrying a mutation in the virulence-regulatory gene *phoP* (KK16), which is known to decrease *S. typhimurium* survival within macrophages (41). An *ntrA::Tn10* strain (KK1) was equally defective for intramacrophage survival. This defect is specifically caused by low-level glutamine synthesis and/or transport, because the addition of the suppressing *glnAp356* mutation to the *ntrA* strain (KK8) restores intramacrophage survival to the wild-type level. Similar results were obtained when glutamine was not added to the culture medium (data not shown). These results potentially identify the macrophage phagolysosome as a low-glutamine host environment in which *S. typhimurium* must grow.

These same strains were tested for their ability to invade HEp-2 cells, a characteristic of pathogenic *S. typhimurium* (32). All strains were equally able to invade HEp-2 cells (less than 3-fold differences in bacterial recovery resulted), while a control strain carrying a mutation in the essential invasion gene *invA* (KK13) was more than 150-fold defective for invasion (data not shown).

**Strains with simultaneous defects in glutamine synthesis and high-affinity transport provide protective immunity against *S. typhimurium*.** Mice were inoculated with various doses of attenuated *S. typhimurium* strains, and survivors were challenged 4 weeks later with the wild-type strain (Table 4). A strain with a  $\Delta(glnA-ntrC)60$  mutation (KK4) served remarkably well as a vaccine. Doses as low as 10 organisms delivered i.p. provided protection against i.p. challenge with  $10^5$  wild-type cells. In large inocula ( $10^5$  i.p.,  $10^9$  oral), this strain is protective against both i.p. and oral wild-type challenges. The

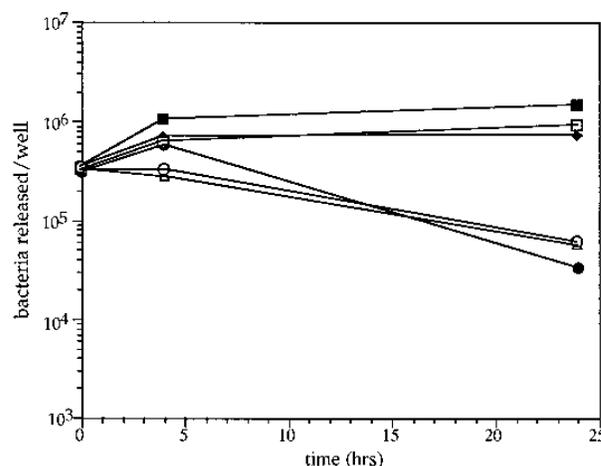


FIG. 3. Survival of *S. typhimurium* strains within J774 macrophages. Macrophages ( $5 \times 10^5$ /well) were infected (see Materials and Methods) with *S. typhimurium* strains 14028 (wild type) (■), KK10 (*ntrB::Tn10*) (□), KK8 (*ntrA::Tn10 glnAp356*) (◆), KK1 (*ntrA::Tn10*) (○), KK4 [ $\Delta(glnA-ntrC)$ ] (▲), and KK16 (*phoP*) (●). The number of viable bacteria at each time point was determined by lysing the macrophages and plating. Results are expressed as the averages of values for three wells. The values with standard deviations for the 24-h time point were as follows: 14028 (wild type),  $1.5 \times 10^6 \pm 3.8 \times 10^5$ ; KK10 (*ntrB::Tn10*),  $9.1 \times 10^5 \pm 2.0 \times 10^5$ ; KK8 (*ntrA::Tn10 glnAp356*),  $7.2 \times 10^5 \pm 1.2 \times 10^5$ ; KK1 (*ntrA::Tn10*),  $6.0 \times 10^4 \pm 4.0 \times 10^3$ ; KK4 [ $\Delta(glnA-ntrC)$ ],  $5.9 \times 10^4 \pm 1.0 \times 10^4$ ; and KK16 (*phoP*),  $3.3 \times 10^4 \pm 9.5 \times 10^3$ .

complete operon deletion  $\Delta(glnA-ntrC)1$  (KK85) also protected against wild-type i.p. challenge at the single large initial inoculum ( $10^5$ ) tested. A  $\Delta glnA ntrA$  double mutant (KK30) gave full protection against wild-type i.p. challenge when administered in small (10) and large ( $10^5$ ) i.p. inocula, whereas a  $\Delta glnA ntrC$  double mutant (KK34) was not as effective in a small initial inoculum (10) but gave good protection when inoculated at  $10^5$  organisms. As a comparison, a strain with a mutation in the virulence-regulatory gene *phoP* (KK16) did not work well as a vaccine; although the strain was highly attenuated, protective immunity resulted in only two of five mice which received a large ( $10^5$ ) initial inoculum.

In large inocula ( $10^5$  i.p.),  $\Delta glnA glnH$  (KK45),  $\Delta glnA glnQ$  (KK50), and  $\Delta glnA \Delta(glnH-P)$  (KK110) double-mutant strains provided full protection against wild-type i.p. challenge. A  $\Delta glnA glnH$  strain (KK45) was fully protective against wild-type i.p. or oral challenge when administered either i.p. or orally.  $\Delta glnA glnH$  and  $\Delta glnA glnQ$  double-mutant strains were less effective when delivered in doses smaller than  $10^3$  i.p. Strains with a single IHF mutation,  $\Delta himD$  (KK26), were attenuated but provided only partial protection against wild-type challenge at a large ( $10^5$ ) inoculum; a  $\Delta glnA \Delta himD$  strain provided no protective immunity against subsequent wild-type challenge at the single large i.p. inoculum ( $10^5$ ).

## DISCUSSION

Our studies have shown that neither prevention of glutamine synthesis nor prevention of high-affinity glutamine transport alone attenuates *S. typhimurium* virulence. Both a glutamine auxotroph ( $\Delta glnA$ ) and mutants defective for high-affinity glutamine transport (*glnH* and *glnQ*) have the same low i.p. LD<sub>50</sub>s (<10) as that of a wild-type strain. However, double mutations of  $\Delta glnA$  and *glnH* or of  $\Delta glnA$  and *glnQ* increase the i.p. LD<sub>50</sub> to  $>10^5$ , similar to a strain defective for the virulence-regulatory protein PhoP. These results demonstrate that an *S. typhi-*

*murium* glutamine auxotroph must acquire glutamine from the host by utilizing the high-affinity glutamine transport system.

A  $\Delta glnA$  strain can be similarly attenuated by mutation of either *ntrA* or *ntrC* or by deletion of the entire *glnA ntrBC* operon. Mutations in *ntrA* and *ntrC* result in decreased transcription of the *glnHPQ* operon, consistent with the attenuated phenotype of these strains resulting from decreased high-affinity glutamine transport. Kustu et al. have previously shown that levels of the periplasmic high-affinity glutamine-binding protein (GlnH) are coregulated with levels of glutamine synthetase, as well as several other amino acid transport systems, through the *ntr* system (31). NtrC and  $\sigma^{54}$ -holoenzyme have further been shown to transcribe the *glnH* promoter of *E. coli* in vitro (6). Our results have extended the characterization of high-affinity glutamine transport in *S. typhimurium* to show that (i) the sequence of the *S. typhimurium glnH* promoter is homologous to that from *E. coli* and shares a consensus  $\sigma^{54}$ -holoenzyme binding site as well as several apparent NtrC binding sites; and (ii) high-level transcription of the *S. typhimurium glnH* promoter requires both  $\sigma^{54}$  and NtrC, as measured by transcriptional *glnHp-lacZ* fusions.

The *E. coli glnH* promoter has also been shown to contain an IHF binding site; IHF facilitates contact between enhancer-bound NtrC and promoter-bound  $\sigma^{54}$ -holoenzyme (6). Although the defined IHF binding site in the *E. coli glnH* promoter and the corresponding sequence in the *S. typhimurium glnH* promoter share only 14 of 27 nucleotides, the consensus binding site for IHF is actually quite small (WATCAAN<sub>4</sub>TTR, where W represents A or T and R represents A or G [7, 20, 33]), and the potential site in the *S. typhimurium* sequence has only one mismatch to the consensus. A mutation in *himD*, which encodes the  $\beta$  subunit of IHF, reduced *glnH* transcription only twofold under our assay conditions, and all strains carrying this mutation were highly attenuated regardless of *glnA* status. Thus, *himD* mutations could not be used in combination with *glnA* deletions to independently assess the role of *glnH* transcription in compensating for the loss of *glnA* function during pathogenesis. Interestingly, the  $\alpha$  subunit of IHF (encoded by *himA*) lies in an operon which was previously identified as being induced within the host during *S. typhimurium* pathogenesis (36), and a polar mutation in this operon also causes attenuation of *S. typhimurium*.

*S. typhimurium* has a low-affinity glutamine transport system with a  $K_m$  of 10  $\mu$ M, in addition to the high-affinity transport system, which has a  $K_m$  of 0.2  $\mu$ M (4); hence, a  $\Delta glnA glnH$  mutant can grow in vitro when supplemented with high levels of glutamine. We presume the attenuation of the  $\Delta glnA glnH$  mutant (as well as the  $\Delta glnA ntrA$  and  $\Delta glnA ntrC$  mutants) is due to slow growth within the host because of limiting amounts of glutamine. As an example, the  $\Delta glnA1$  mutant grows with the same doubling time ( $\sim$ 60 min) in minimal NH<sub>4</sub> medium whether supplemented with 2 mM or 200  $\mu$ M glutamine. This growth rate is only slightly slower than that of the wild type in the same medium ( $\sim$ 55 min). In contrast,  $\Delta glnA ntrA$ ,  $\Delta(glnA-ntrC)$ , and  $\Delta glnA glnH$  strains grow more slowly in 200  $\mu$ M glutamine (doubling times of 155, 135, and 160 min, respectively) than in 2 mM glutamine (doubling times of 110, 90, and 110 min, respectively). The requirement for higher levels of glutamine to sustain faster growth of these three strains explains their virulence defect if in fact the level of glutamine available in animal tissues and fluids is low.

*S. typhimurium* is known to reside within the phagolysosome of macrophages, and macrophage survival is essential for virulence (3, 12). Notably, the  $\Delta(glnA-ntrC)$  strain is defective for survival within J774 macrophages, similar to a strain carrying a mutation in the virulence-regulatory gene *phoP* which is known

to cause a defect in macrophage survival. An *ntrA* strain is similarly defective for macrophage survival, but this defect is completely overcome by an increase in *glnA* transcription with the addition of a *glnAp356* mutation. The growth rate of *S. typhimurium* under nitrogen-limiting conditions is determined by the internal pool size of glutamine (21), indicating that the defect in these strains is due to slow growth caused by low internal levels of glutamine and thereby demonstrating that the phagolysosome of the cultured J774 macrophage is a low-glutamine environment. If we extrapolate from these in vitro results, we would predict that the phagolysosomes of host macrophages also contain low levels of glutamine, thus explaining the attenuation of strains with simultaneous defects in glutamine synthesis and high-affinity transport. The phagolysosome containing *S. typhimurium* acidifies to a pH of  $<5.0$  (1). Since glutamine is known to be acid labile, acidification of the phagolysosome may result in a decrease in available glutamine, making the phagolysosome particularly glutamine deficient. Alternatively, glutamine may be actively excluded from the phagolysosome. Harms et al. determined the amount of in vivo free amino acids within lysosomes of whole rat liver (17) but were unable to distinguish between glutamate and glutamine due to the acid lability of glutamine. Thus, it remains to be determined whether all host compartments or just some important subset, like the phagolysosome, has such low levels of available glutamine that high-affinity transport is required to scavenge it.

$\sigma^{54}$  is required for the transcription of virulence genes in a number of organisms. In *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*, it transcribes pilin genes necessary for adherence to host epithelial cells (23, 25); in *Vibrio anguillarum*, it is required for colonization of the fish host, which may be partially due to its required role in the motility of this organism (47); and in *Vibrio cholerae*, it is required for normal colonization and also for motility, which in turn probably plays a role in colonization and dissemination of this pathogen (26). Our results preclude the involvement of  $\sigma^{54}$  in the transcription of any essential *Salmonella* virulence gene; rather, the role of  $\sigma^{54}$  in the pathogenesis of *S. typhimurium* is to coordinate the transcription of glutamine synthesis and high-affinity transport genes to ensure an adequate supply of this crucial amino acid. The only virulence defect of an *ntrA* strain was seen in a competition assay, and this effect was almost completely overcome in an *ntrA glnAp356* strain, indicating that the only virulence defect in an *ntrA* strain is low *glnA* transcription. The same effect was seen in intramacrophage survival; an *ntrA* strain appears as defective for survival within the phagolysosome as a  $\Delta(glnA-ntrC)$  strain, which might suggest that an *ntrA* strain should be attenuated in a manner similar to a  $\Delta(glnA-ntrC)$  strain. However, the *ntrA* strain showed no defect in an LD<sub>50</sub> assay, even though this strain is a glutamine auxotroph under laboratory conditions, suggesting that the secondary promoters upstream of the  $\sigma^{54}$ -dependent promoters of *glnA* and *glnH* may be expressed at higher levels during in vivo growth in the host.

Strains with the  $\Delta glnA$  mutation combined with mutations in either *ntrC*, *ntrA*, or *glnH* provided protection against subsequent challenge with wild-type *S. typhimurium* and show promise as live attenuated vaccine strains. The  $\Delta(glnA-ntrC)$  mutation provided the best protection at both low and high doses when delivered both i.p. and perorally. This type of metabolic attenuating mutation has the benefit of not affecting virulence gene expression, which may provide useful antigens for recognition by the immune system. Moreover, although the  $\Delta glnA glnH$  strain needed to be inoculated in slightly higher doses for full protection against wild-type challenge, the *ntr* system is

unaffected by these mutations and could be used to drive high-level transcription of heterologous antigens in a live vaccine strain simply by the provision of an *ntn*-regulated promoter. In principle, attenuating an auxotroph by disrupting the transport mechanism needed to supply the auxotrophy could be a useful approach to developing vaccines for other pathogenic organisms. The specific example of mutations affecting glutamine synthesis and high-affinity transport should be investigated in different organisms (e.g., intracellular and extracellular pathogens). The general concept can be studied by constructing other biosynthesis-transport paired mutations or, alternatively, by disrupting transport genes in naturally occurring auxotrophic pathogens which rely on scavenging host nutrients.

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