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Roles of SpoT and FNR in NH_4^+ Assimilation and Osmoregulation in GOGAT (Glutamate Synthase)-Deficient Mutants of *Escherichia coli*

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An osmosensitive mutant of *Escherichia coli* was isolated and shown to harbor two mutations that were together necessary for osmosensitivity. One (*ossB*) was an insertion mutation in the *gltBD* operon, which encodes the enzyme glutamate synthase (GOGAT), involved in ammonia assimilation and L-glutamate biosynthesis. The other (*ossA*) was in the *fnr* gene, encoding the regulator protein FNR for anaerobic gene expression. Several missense or deletion mutations in *fnr* and *gltBD* behaved like *ossA* and *ossB*, respectively, in conferring osmosensitivity. A mutation affecting the DNA-binding domain of FNR was recessive to *fnr*$^+$ with respect to the osmotolerance phenotype but was dominant-negative for its effect on expression of genes in anaerobic respiration. Our results may most simply be interpreted as suggesting the requirement for monomeric FNR during aerobic growth of *E. coli* in high-osmolarity media, presumably for L-glutamate accumulation via the GOGAT-independent pathway (catalyzed by glutamate dehydrogenase [GDH]), but the mechanism of FNR action is not known. We also found that the *spoT* gene (encoding guanosine 3′,5′-bispyrophosphate [ppGpp] synthetase II/ppGpp-3′ pyrophosphohydrolase), in multiple copies, overcomes the defect in NH_4^+ assimilation associated with GOGAT deficiency and thereby suppresses osmosensitivity in *gltBD fnr* strains. Enhancement of GDH activity in these derivatives appears to be responsible for the observed suppression. Its likely physiological relevance was established by the demonstration that growth of *gltBD* mutants (that are haploid for *spoT*$^+$) on moderately low [NH_4^+] was restored with the use of C sources poorer than glucose in the medium. Our results raise the possibility that SpoT-mediated accumulation of ppGpp during C-limited growth leads to GDH activation and that the latter enzyme plays an important role in N assimilation in situ hitherto unrecognized from studies on laboratory-grown cultures.

The biosynthesis of L-glutamate is intimately associated with N assimilation in *Escherichia coli* and *Salmonella typhimurium* (43) and is achieved through two independent pathways catalyzed, respectively, by (i) glutamate dehydrogenase (GDH), encoded by *gdhA*, and (ii) glutamine synthetase in combination with glutamate synthase (GOGAT). In low-[NH_4^+] medium, the latter is the only pathway that contributes to N assimilation (42, 43). The two subunits of GOGAT are encoded by the *gltB* and *gltD* genes, which are organized (along with a third gene, *gltF* [7, 8]) as an operon at 70 min on the linkage map.

The cytoplasmic concentration of L-glutamate is elevated in cells grown at high osmolarity, where it is believed to serve as a counterion to K^+, which also accumulates under these conditions (38; reviewed in reference 12). L-Glutamate accumulation in high-osmolarity-grown cells occurs through increased synthesis. With high exogenous [NH_4^+], L-glutamate accumulation at high osmolarity is unaffected by single mutations that block either one or the other biosynthetic pathway (2, 13, 37). Botsford et al. (2) and McLaggan et al. (37) have argued that the increase in L-glutamate pools in cultures grown at elevated osmolarity represents a very small, and possibly insignificant, load in comparison with the total biosynthetic flux through the amino acid. On the other hand, Csonka et al. (13) have recently shown that, in GOGAT-defective mutants of *S. typhimurium* grown under ammonia-limiting conditions, there is an inverse relationship between growth rate and the osmolarity of the culture medium. The latter result provides indirect genetic evidence that increased L-glutamate synthesis is necessary for optimal growth under hyperosmotic stress.

Regulation of NH_4^+ assimilation and glutamate synthesis through the glutamine synthetase-GOGAT pathway is tied in with the complex cascadal system of nitrogen regulation, referred to as Ntr (43). Information on regulation of the GDH pathway is more limited, and there appear also to be significant species differences in this regard (24, 43, 52). Synthesis of both GOGAT and GDH is feedback repressed by glutamate (43, 52). GDH activity in vitro is also stimulated by K^+ (38), a finding which has been interpreted as the possible basis for increased glutamate levels in high-osmolarity-grown cultures. However, Ohyama et al. (41) have shown that glutamate accumulation at elevated osmolarity is unaffected even in cells that fail to accumulate K^+.

In this report, we describe the identification of a *gltBD* mutant derivative of *E. coli* (in which glutamate synthesis can proceed only via GDH) that is osmosensitive even at high [NH_4^+]. The data from experiments using this mutant suggest that two known regulatory genes, *fnr* (earlier characterized for its role in anaerobic gene regulation [20, 32, 51]) and *spoT* (involved in the metabolism of guanosine 3′,5′-bispyrophosphate [ppGpp] [6, 15, 25, 53]) may each have a role in regulation of the GDH pathway in aerobically grown cultures, both at high osmolarity and during C-limited growth.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. All bacterial strains were derivatives of *E. coli* K-12 and are listed in Table 1. Phage P1*kc* was from our laboratory stocks. λp1(209) phage was obtained from M. J. Casadaban. The phages λNK370, λ1098, and λ1105, used as vectors for the transposition of Tn*10*, Tn*10*dTet, and Tn*10*dKan, respectively, were obtained from N. Kleckner. A

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TABLE 1. *E. coli* K-12 strains

Strain	Genotype[a]	Source or reference
AB1157 (Mu *c*ts)[b]	*thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4 rpoS338 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1* (Mu *c*ts)	R. Jayaraman
CAG12153	*zha-6*::Tn*10*	49
CF1648	Wild-type	Same as MG1655 (53)
CSH57	*purE trp his argG ilv leu met thi ara lac xyl mtl rpsL*	16
JM101	*supE thi* Δ(*gpt-lac*)*5* F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	40
JP2144	*his-29*(Am) *trpA9605*(Am) *ilv-1 tyrR366*	9
JP2769[c]	*his-29*(Am) *ilv-1 zcj-352*::Tn*10*	9
JP3301	F$^+$ *purE trp his argG ilv leu met thi ara xyl mtl rpsL* Δ(*argF-lac*)*U169 pheR362 recA56* [λp(*pheA-lac*)]	18
JRG861-b	*fnr-8*	48
KL14[d]	Hfr(PO68) *thi-1 relA1 spoT1 fnr-267*	B. J. Bachmann
MAL103	*araB*::Mu *c*ts *araD139* Δ(*gpt-lac*)*5 rpsL* [Mu d1(*lac* Ap)]	5
MC4100	Δ(*argF-lac*)*U169 rpsL150 araD139 relA1 flbB5301 deoC1 ptsF25 rbsR*	5
PA340	*thr-1 fhuA2 leuB6 lacY1 supE44 gal-6 gdh-1 hisG1 rfbD1 galP63* Δ*gltBDF500 rpsL9 malT1 xyl-7 mtl-2 argH1 thi-1*	B. J. Bachmann
RZ8480	*lacZ*Δ145 Δ*fnr*::Ω(Sm^r Sp^r) *narG234*::Mu dI1734(*lac* Kan)	32
GJ193[e]	MC4100 *fnr-266 gltBD238*::Mu d1(*lac* Ap)	This study
GJ309	GJ193 *fnr*$^+$ *zda-900*::Tn*10*	This study
GJ311	GJ193 *fnr*$^+$ *zda-901*::Tn*10*	This study
GJ312	GJ193 *zda-901*::Tn*10*	This study
GJ395	MC4100 *fnr-266 zda-901*::Tn*10*	This study
GJ396[f]	MC4100 *fnr-266* Δ(*zda-901*::Tn*10*)*461*	This study
GJ901	GJ193 *gltBD*$^+$ *zha-900*::Tn*10*dTet	This study
GJ910	GJ193 *gltBD*$^+$ *zha-901*::Tn*10*dKan	This study
GJ912	GJ193 *zha-901*::Tn*10*dKan	This study
GJ913	GJ396 *gltBD238*::Mu d1(*lac* Ap) *zha-901*::Tn*10*dKan	This study
GJ920	GJ396 *zha-901*::Tn*10*dKan *gltBD238*::*lac* [stabilized by λp1(209)]	This study
GJ921	MC4100 *zha-901*::Tn*10*dKan *gltBD238*::*lac* [stabilized by λp1(209)]	This study
GJ930	GJ921 *fnr-8 zcj-352*::Tn*10*	This study
GJ946	GJ396 Δ*gltBDF500 zha-6*::Tn*10*	This study
GJ959	GJ921 Δ*fnr*::Ω(Sm^r Sp^r)	This study
GJ966	MC4100 Δ*fnr*::Ω(Sm^r Sp^r) Δ*gltBDF500 zha-6*::Tn*10*	This study
GJ967	MC4100 Δ*gltBDF500 zha-6*::Tn*10*	This study
GJ968	MC4100 Δ*fnr*::Ω(Sm^r Sp^r)	This study
GJ970	GJ921 *fnr-267 zcj-352*::Tn*10*	This study
GJ971[g]	GJ193 *recA srl*::Tn*10*	This study
GJ972	GJ311 *narG234*::Mu dI1734(*lac* Kan)	This study
GJ973	GJ396 *narG234*::Mu dI1734(*lac* Kan)	This study
GJ1235	MC4100 *argR203*	G. UmaPrasad

[a] Genetic nomenclature is as in Bachmann (1). All strains are F$^-$ unless otherwise indicated. Allele numbers are indicated where they are known. Allele numbers for new mutations identified in this laboratory were provided by Barbara Bachmann (*E. coli* Genetic Stock Center).

[b] The position of the Mu insertion in AB1157 (Mu *c*ts) is not known. The presence of the *rpoS338* mutation in AB1157 was identified for the first time in this laboratory (36).

[c] The insertion previously designated *zci-2*::Tn*10* (9) has now been redesignated as *zcj-352*::Tn*10* (42a).

[d] The presence of the *fnr-267* mutation in KL14 was identified for the first time in the present study.

[e] *fnr-266* and *gltBD238*::Mu d1(*lac* Ap) are redesignations, respectively, of the *ossA* and *ossB* mutations in GJ193, described in the text. GJ193 also carries an additional Mu d1(*lac* Ap) insertion at an undetermined site, as indicated by the fact that *gltBD*$^+$ transductants of GJ193 (such as GJ910) continue to be Amp^r and temperature sensitive.

[f] GJ396 was obtained as a spontaneous Tet^s mutant of GJ395.

[g] GJ971 was constructed by transduction of *recA* linked to *srl*::Tn*10* from strain GJ216 (26) into GJ193.

lysate of the phage Mu d1(*lac* Ap) was prepared by temperature induction of MAL103, as described previously (5). The phage Mu d5005 lysate used in the in vivo cloning experiments was prepared by temperature induction of an AB1157 Mu *c*(Ts) derivative carrying the plasmid pEG5005, as described previously (19).

The plasmid vectors pACYC184 (40), pBR329 (11), and pCL1920 (33) have been described. The recombinant plasmids pGS24 (carrying the *fnr*$^+$ gene in plasmid vector pBR322 [48]), pHX41 (carrying the minimal *spoT*$^+$ gene expressed from an upstream *lac* promoter on an Amp^r plasmid vector [53]), and pRZ7411-EK209 (carrying the dominant-negative EK209-*fnr* mutation in vector pACYC184 [32]) were obtained from J. Guest, M. Cashel, and B. Lazazzera, respectively. Plasmid pHYD819 was constructed in this study by subcloning the *Bam*HI-*Hin*dIII fragment carrying the *fnr*$^+$ gene from pGS24 into the appropriate sites in the pSC101-derived plasmid pCL1920. Other plasmids that were constructed in this study are described in the text.

Growth media and antibiotics. Glucose-minimal A medium (supplemented with the appropriate auxotrophic requirements) and LB were routinely used as defined and nutrient media, respectively (40). Solid media were prepared by the addition of 1.5% agar (Difco). Thioglycolate broth medium (with hemin and vitamin K) was obtained from HiMedia Laboratories (Bombay, India) and was used at 3% with 0.4% glucose supplementation. Growth on various N sources was tested using W salts basal medium (50), to which was added (as a C source) glucose or other indicated substances at 0.4% and (as an N source) NH_4Cl at the desired concentration or any of the indicated amino acids at 0.2%. Unless otherwise specified, antibiotics were used at the following final concentrations (micrograms per milliliter): ampicillin, 50; chloramphenicol, 25; kanamycin, 50; spectinomycin, 50; streptomycin, 50; and tetracycline, 15.

Tests of osmotolerance and osmosensitivity. The relative osmotolerance of strains was determined by streaking them on the surface of glucose-minimal A plates supplemented with various concentrations of NaCl and optionally with 1 mM glycine betaine or L-proline or by inoculating them in broth of the same composition. Phenotypes for all strains reported in this study are based on comparisons between isogenic derivatives that show no discernible growth differences in media not supplemented with NaCl. Typically, the osmotolerant strains grow to form single colonies in 42 to 60 h at 37°C on 0.45 M NaCl or on 0.7 M NaCl supplemented with glycine betaine or L-proline, whereas the osmo-

sensitive mutants do not grow even after 80 h on these media. Measurements of doubling time in broth cultures correlated well with the plate phenotype.

Isolation of osmosensitive mutant GJ193. The procedure of Casadaban and Cohen (5) was followed to obtain a population of clones carrying random transpositions of phage Mu d1(*lac* Ap) in the chromosome of strain MC4100. This population was inoculated at 10^7 cells per ml into 10 ml of glucose-minimal A medium supplemented with 0.7 M NaCl and 1 mM glycine betaine and incubated at 30°C for 4 h before ampicillin was added to a final concentration of 2 mg/ml. It is known that Mu d1(*lac* Ap) lysogens are sensitive to growth in the presence of ampicillin at this high concentration (31). Surviving cells after a further 4-h incubation were harvested by filtration and grown overnight in glucose-minimal A medium, and the cycle of ampicillin enrichment was repeated. The culture was then plated on glucose-minimal A medium, and individual colonies were tested for osmosensitivity. GJ193 was identified as a Lac^- mutant in this screen that was significantly less osmotolerant than MC4100.

Growth at various pO_2s and tests for Fnr^- phenotype. The growth obtained on plates and in broth following routine microbiological practices is referred to in this study as growth under ordinarily aerobic conditions. Anaerobic growth was achieved by either (i) inoculation and growth of strains in thioglycolate broth or (ii) incubation of plates in a vacuum desiccator that had been tightly sealed after evacuation. The efficacy with which anaerobiosis was achieved under both conditions was monitored by assaying the activation of *narG*::*lac* expression in an fnr^+ strain (with 20 mM KNO_3 as an inducer) and also by the test of methylene blue reduction in the desiccator. Hyperaerobic conditions refer to growth on plates in a sealed vacuum desiccator (as above) that had been flushed and filled with O_2 to about 0.8 atm (1 atm = 101.29 kPa) after initial air evacuation.

Two tests of the Fnr^- phenotype were employed in this study: (i) inability to reduce 5 mM sodium nitrite added to thioglycolate broth, with residual nitrite being detected by the qualitative color reaction described by Cole and Ward (10); and (ii) reduced expression of the nitrate reductase operon, measured with the aid of a chromosomal *narG*::*lac* fusion during anaerobic growth in medium supplemented with 20 mM KNO_3 (32).

Transposon techniques. Random transpositions of Tn*10*, Tn*10*dTet, or Tn*10*dKan were obtained following infection of strains with the corresponding phage vectors, as described previously (40). P1*kc* lysates prepared on each population of Tet^r or Kan^r clones were then used in transductions into the osmosensitive mutant strain with simultaneous selection for the transposon marker and for the osmotolerance phenotype (on glucose-minimal A plates supplemented with 0.7 M NaCl and 1 mM glycine betaine). P1 lysates prepared on each of the colonies that grew on the double-selection plates were then used to examine the extent of linkage between transposon insertion and the locus involved in osmotolerance and to obtain strains in which the transposon was now linked to the mutant gene.

Insertions of Tn*1000* (γδ) into plasmids were obtained and physically mapped as described by Guyer (21).

Enzyme assays. The specific activity of β-galactosidase was measured, after permeabilization of cells with sodium dodecyl sulfate-chloroform, by the method of Miller (40), and the values are expressed in units defined therein. In some experiments, colonies growing on solid media were scraped off and suspended in minimal A buffer for β-galactosidase assays.

Cell extracts for determination of specific activities of GOGAT and GDH were prepared as described previously (17), with the modification that harvested cells, after being washed, were resuspended in 3 ml of ice-cold 50 mM Tris-Cl buffer (pH 7.6) plus 10 mM β-mercaptoethanol prior to passage through a French pressure cell. The extracts were stored on ice and used for enzyme activity measurements within 3 h. GOGAT and GDH activities were measured (after correction for endogenous NADPH oxidase activity) essentially as described by Meers et al. (39), with the modification that the reactions were performed at room temperature in a total volume of 1 ml. Protein concentrations in cell extracts were determined by the method of Bradford (3). Enzyme specific activities are expressed as milliunits per milligram of protein in the cell extracts, where 1 U is defined as the amount of enzyme required to oxidize 1 μmol of NADPH (extinction coefficient at 340 nm, 6220 M^{-1} cm^{-1}) per min at room temperature.

Recombinant DNA techniques. The protocols of Sambrook et al. (45) were followed for experiments with recombinant DNA, random-primer labeling of plasmid DNA with [α-^{32}P]dATP (purchased from BRIT, Department of Atomic Energy, Bombay, India), and hybridization to DNA blotted on membrane filters. Nylon membranes carrying immobilized lambda phage DNA from the ordered *E. coli* genomic library (29) were obtained from Takara Shuzo Company, Kyoto, Japan.

Other techniques. The procedures for conjugation (40) and P1*kc* transduction (16) were as described previously. Spontaneous Tet^s derivatives of Tet^r strains were selected by the protocol of Maloy and Nunn (35). The method of Komeda and Iino (30) was used to convert the Mu d1(*lac* Ap) lysogen GJ913 into a temperature-resistant, Amp^s derivative, GJ920, which now carries a λp1(209) prophage insertion at the mutant locus.

RESULTS

Osmosensitivity of GJ193 and identification of two mutant loci, *ossA* and *ossB*. The osmosensitive strain GJ193 was isolated following Mu d1(*lac* Ap) mutagenesis of MC4100 and ampicillin enrichment as described above. In comparison with MC4100, GJ193 exhibited impaired growth on NaCl-containing high-osmolarity plates both without and with supplementation with glycine betaine or L-proline (data not shown, but see Fig. 1). Growth of GJ193 was also inhibited on medium rendered hyperosmolar by addition of sucrose (data not shown), indicating that the inhibition was not chemical or ion specific. Glycerol, which is a freely permeable solute, did not affect the growth of GJ193. By introducing mutations in other osmoregulatory genes (12) such as *proU*/*proP* and *otsA* into both MC4100 and GJ193, we were able to demonstrate that expression of the osmosensitive phenotype in GJ193 is mediated by osmoregulatory mechanism(s) independent, respectively, of glycine betaine or L-proline uptake or trehalose biosynthesis (data not shown).

Transposon tagging experiments were used to demonstrate that GJ193 has two mutations, *ossA* and *ossB*::Mu d1(*lac* Ap), that are together necessary for osmosensitivity. A P1*kc* lysate prepared on a population of Tn*10*-mutagenized MC4100 clones was used to transduce GJ193 simultaneously to Tet^r and osmotolerance. Two of the colonies so obtained had Tet^r insertions (designated *zda-900*::Tn*10* and *zda-901*::Tn*10* based on the mapping data below) 99 and 50% cotransducible, respectively, with the MC4100 locus designated $ossA^+$. Whereas $ossA^+$ transductants of GJ193 were significantly osmotolerant, *ossA* derivatives of MC4100 obtained in the reverse transductional cross were not osmosensitive, indicating that the *ossA* mutation was necessary but not sufficient for exhibition of this phenotype. The MC4100 *ossA* transductants also continued to be Amp^s and Mu sensitive, indicating that the concerned mutation was not a Mu d1(*lac* Ap) insertion.

Random transpositional insertions of Tn*10*dTet or of Tn*10*dKan were then separately generated into the chromosome of GJ396 (an *ossA* derivative of MC4100), and P1 lysates prepared on pools of such clones were used again to transduce GJ193 simultaneously to Tet^r or Kan^r (as appropriate) and to osmotolerance. In this manner, one Tn*10*dTet and one Tn*10*dKan insertion allele (designated *zha-900*::Tn*10*dTet and *zha-901*::Tn*10*dKan, respectively) were identified that were almost 100% linked to one another and that were each in turn 80% linked to a gene (designated $ossB^+$) in GJ396 that by itself conferred osmotolerance upon introduction into GJ193. A pair of isogenic Tn*10*dKan derivatives of GJ193 carrying either the wild-type or the mutant *ossB* allele at this locus were designated GJ910 and GJ912, respectively.

When GJ912 was used as the donor in transduction into the *ossA* mutant GJ396, approximately 5% of the Kan^r colonies had now become as osmosensitive as the original mutant, GJ193 (data not shown). These colonies (one representative designated GJ913) were also Amp^r, Mu immune, and temperature sensitive for growth and remained Lac^-, permitting the following conclusions: (i) the mutation at *ossB* in GJ193 is caused by Mu d1(*lac* Ap) insertion in the antisense orientation, and this explains the observed discrepancy in cotransduction frequency of the Kan^r insertion with $ossB^+$ (80%) and with *ossB*::Mu d1(*lac* Ap) (5%) (both because of the large size of the prophage and of zygotic induction occurring in the latter cross); and (ii) mutations at each of the two identified loci, *ossA* and *ossB*, in GJ193 are necessary and sufficient for exhibition of the osmosensitive phenotype.

The availability of the linked transposon markers enabled

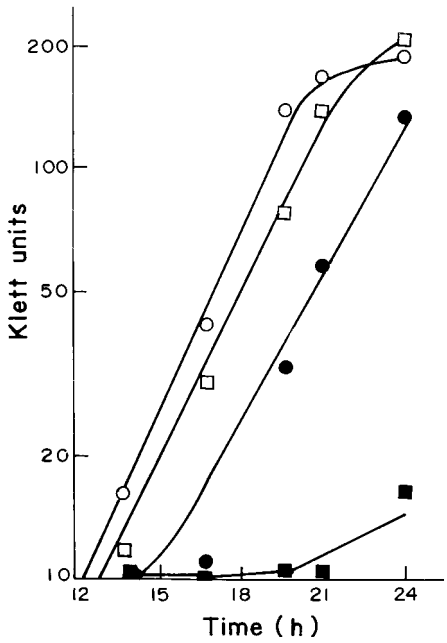

FIG. 1. Growth of isogenic *ossA* (*fnr*) *ossB* (*gltBD*) strains in high-osmolarity medium. Cultures of the different strains grown to stationary-phase in glucose-minimal A medium were inoculated into glucose-minimal A medium supplemented with 0.7 M NaCl and 1 mM glycine betaine, and optical density (Klett units) for each was monitored as a function of time of incubation in a shaker water bath at 37°C. Strain symbols (relevant genotypes and initial inoculum ratios in parentheses): ○, MC4100 (*fnr*$^+$ *gltBD*$^+$, 1:1,000); □, GJ396 (*fnr gltBD*$^+$, 1:750); ●, GJ921 (*fnr*$^+$ *gltBD*, 1:250); and ■, GJ920 (*fnr gltBD*, 1:250).

the construction, by P1*kc* transduction, of defined isogenic *ossA* (GJ396), *ossB* (GJ921), and *ossA ossB* (GJ920) derivatives of the wild-type strain MC4100. [The *ossB*::Mu d1(*lac* Ap) allele used in these constructions had earlier been stabilized by the method of Komeda and Iino (30).] Tests of osmotolerance on this isogenic panel of four strains, both on plates (data not shown) and in broth (Fig. 1), indicated that both mutations are necessary for pronounced osmosensitivity. The mutant defective in *ossB* alone was moderately osmosensitive, whereas that defective in *ossA* alone was no different from the wild type.

Standard techniques of conjugational and transductional mapping (49) were used to place the *ossA* and *ossB* loci to 29.5 and 70 min, respectively, on the *E. coli* chromosome. With the aid of several three-factor crosses, the gene order (reading clockwise) at each of the two loci was established to be *trp-zcj-352*::Tn*10-tyrR*-(*ossA-zda-900*::Tn*10*)-*zda-901*::Tn*10* and *argG-ossB*-(*zha-900*::Tn*10*dTet-*zha-901*::Tn*10*dKan)-*zha-6*::Tn*10-argR* (data not shown; see Fig. 2). On the basis of their map locations (1), we decided to examine whether *ossA* and *ossB* are alleles of *fnr* and *gltBD*, respectively.

Mapping of *ossB* to the *gltBD* locus. The *gltB* and *gltD* genes at 70 min encode the large and small subunits, respectively, of GOGAT, the enzyme required for NH_4^+ assimilation and L-glutamate biosynthesis, particularly when the availability of NH_4^+ in the medium is low (43). The following lines of evidence indicate that *ossB* is an insertion in *gltBD*.

(i) Growth of *gltBD* mutants is either abolished or substantially reduced on W salts medium supplemented with <1 mM NH_4^+ or with any of a variety of amino acids, including glycine, L-arginine, L-ornithine, L-histidine, L-proline, or L-alanine, as the sole N source (42). We could show that GJ193 and all derivatives that carried the *ossB* mutation, including GJ913, GJ920, and GJ921, were Glt$^-$ in that they did not grow on the media above (see Table 4), whereas isogenic *ossB*$^+$ derivatives were Glt$^+$.

(ii) Cell extracts prepared from GJ920 also displayed negligible GOGAT activity and normal GDH activity (the alternative enzyme for glutamate biosynthesis at high [NH_4^+]) compared with the levels in MC4100 (Table 2).

(iii) Strain GJ946, which was constructed by introducing a known Δ*gltBDF500* mutation (from strain PA340) into the *ossA ossB*$^+$ strain GJ396, was also osmosensitive (data not shown).

(iv) The osmosensitivity of GJ193 (relative to MC4100) was completely alleviated on high-osmolarity plates supplemented with 0.2% L-aspartate or L-asparagine (data not shown). It is known that, at this concentration, either supplement can bypass the need for GOGAT in N assimilation (42, 43).

(v) Finally, as described below, a recombinant plasmid from an *E. coli* genomic library was obtained that complemented GJ193 for both osmosensitivity and the ability to grow on low-[NH_4^+] medium. We therefore conclude that the *ossB* muta-

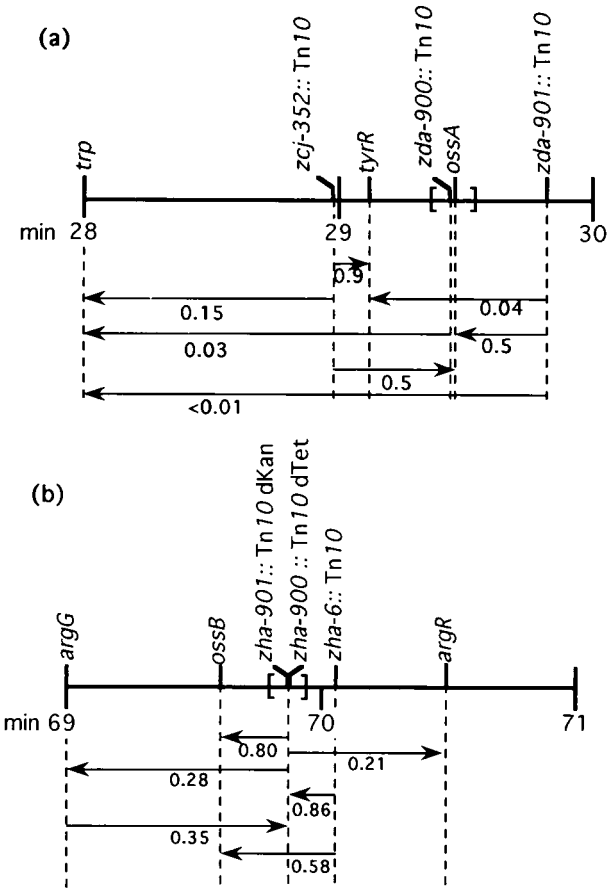

FIG. 2. Transductional mapping data for *ossA* (a) and *ossB* (b) loci. Each two-factor cross is represented by an arrow (base, selected marker; arrowhead, unselected marker), and the cotransduction frequency value is marked beneath the arrow. Not marked in the figure is the value (0.99) for the cross involving *zda-900*::Tn*10* (selected) and *ossA* (unselected). The deduced order and positions of markers are indicated on the 28- to 30-min (a) and 69- to 71-min (b) regions of the *E. coli* linkage map. Where the inter se order of markers is not known, their positions are bracketed. Sources for the different alleles used in the mapping (and not described in the text) are as follows: *trp* and *argG*, CSH57; *zcj-352*::Tn*10*, JP2769; *tyrR366*, JP2144; *zha-6*::Tn*10*, CAG12153; and *argR203*, GJ1235.

TABLE 2. GDH and GOGAT activities in *ossA* (*fnr*) and *ossB* (*gltBD*) derivatives[a]

Strain	Genotype	Sp. act. (mU/mg of protein)	
		GDH	GOGAT
MC4100	Wild-type	1,090	143
GJ396	*fnr-266*	1,140	ND[b]
GJ920	*fnr-266 gltBD238*	1,370	<10
GJ946/pBR329	Δ*gltBD fnr-266*/vector	1,250	<10
GJ946/pHYD809	Δ*gltBD fnr-266*/multicopy *spoT*$^+$	3,170	22

[a] Enzyme specific activities were determined in cell extracts of cultures grown to mid-log phase at 37°C in 0.4% glucose–2 mM NH_4^+–W salts medium with antibiotic supplementation as appropriate.
[b] ND, not determined.

tion is an insertion in the *gltBD* locus, and we have accordingly designated it *gltBD238*::Mu d1(*lac* Ap).

***ossA* is allelic to *fnr*.** The *fnr* gene product is a positive activator of several anaerobically expressed genes, including those for nitrite reductase, nitrate reductase, formate dehydrogenase, formate reductase, and several anaerobic hydrogenases in *E. coli* (20, 51). The following results established that *ossA* is a mutation in *fnr*, which we have designated *fnr-266.*

(i) A qualitative test for nitrite reductase activity (10) indicated that GJ193 and all *ossA* derivatives, including GJ312, GJ396, and GJ913, were deficient for this enzyme under conditions in which the isogenic control strains could express it.

(ii) Strains GJ930 and GJ959, which were constructed by introducing the *fnr-8* and Δ*fnr*::Ω mutations from JRG861-b and RZ8480, respectively, into the *ossA*$^+$ *gltBD* strain GJ921, were osmosensitive (data not shown). Likewise, the Δ*gltBDF* Δ*fnr* double mutant GJ966 exhibited a synthetic osmosensitivity phenotype, whereas the corresponding single mutation strains GJ967 and GJ968 were osmotolerant (data not shown).

(iii) The *ossA* mutation in strain GJ973 was also associated with reduced anaerobic expression of a *narG*::*lac* fusion (see Table 3), to the same extent as that obtained with the previously characterized *fnr-8* or Δ*fnr*::Ω mutations (data not shown).

(iv) Plasmids pGS24 and pHYD819 are derivatives, respectively, of pBR322 (10 to 15 copies per cell) and pSC101 (4 to 6 copies per cell), which carry the *fnr*$^+$ gene. pGS24 (or pHYD819) transformants of both GJ920 (*ossA gltBD*) and GJ921 (*ossA*$^+$ *gltBD*) exhibited equivalent levels of osmotolerance, suggestive of a positive complementation result (although it may be noted that the degree of osmotolerance of all the transformants was somewhat less than that of untransformed GJ921 itself, possibly because of toxicity associated with increased *fnr*$^+$ gene dosage) (data not shown).

(v) Finally, in the course of our studies, we discovered that the commonly used Hfr strain KL14 bears a mutation at 29.5 min that we have designated *fnr-267*, which confers an Fnr$^-$ phenotype and which confers osmosensitivity in a *gltBD* background (GJ970; data not shown).

In light of the evidence (discussed below) for the synergism between the *gltBD* and *fnr* mutations, we examined whether the specific activity of GDH is altered in the *fnr* mutants. The results in Table 2 indicate that *fnr* mutants possess normal GDH activity after growth in W salts medium. Even after growth in high-osmolarity medium (W salts medium supplemented with 0.6 M NaCl and 1 mM glycine betaine), there was no difference in GDH specific activity between *fnr*$^+$ and *fnr* derivatives (values of 718 and 710 mU/mg of protein, respectively, for MC4100 and GJ396).

Effects of pO_2 and of the negative-dominant *fnr* mutation on osmotolerance. Although FNR has earlier been characterized as an anaerobic regulator protein, the *fnr*-associated osmosensitivity phenotype obtained in this study was manifested even on plates incubated under ordinarily aerobic growth conditions. Lazazzera et al. (32) have provided evidence that the FNR protein is inactive as a monomer in aerobically grown cultures and active as a dimer during anaerobic growth. They have also identified several mutations in the region encoding the DNA-binding domain of FNR that are dominant-negative over *fnr*$^+$ because of the presumed formation of inactive mixed dimers; one such mutant is FNR-EK209 (with a glutamate-to-lysine substitution at position 209 in the protein). We reasoned that if FNR was able to function even as a monomer in osmoregulation, *gltBD* strains that are merodiploid *fnr*$^+$/*fnr*-EK209 would remain osmotolerant. In the experiments described below, we used the expression of a *lac* fusion to the nitrate reductase operon (*narG*::*lac*) as a quantitative measure of the concentration of functional dimeric FNR species in the different strains and under different growth conditions (32).

In the *gltBD fnr* strain background (GJ193), a plasmid carrying the *fnr*-EK209 mutant gene conferred an osmosensitive phenotype (Table 3). We then introduced the same plasmid (and, separately, the plasmid vector pACYC184 as control) into strain GJ972 (which is *gltBD fnr*$^+$ and carries the *narG*::*lac* fusion) and tested the derivatives for both osmotolerance and β-galactosidase expression under anaerobic, ordinarily aerobic, or hyperaerobic growth conditions (Table 3). As expected, the magnitude of *narG*::*lac* expression in the GJ972 derivatives

TABLE 3. *fnr* and pO_2 effects on *narG*::*lac* expression and growth at high osmolarity[a]

Strain derivative (chromosomal genotype)	Plasmid	Anaerobic		Ordinarily aerobic		Hyperaerobic	
		β-Gal	Growth	β-Gal	Growth	β-Gal	Growth
GJ972 (*gltBD fnr*$^+$)[b]	pACYC184	310	++	72	++	21	++
GJ972 (*gltBD fnr*$^+$)	pRZ7411-EK209	28	++	18	++	9	++
GJ973 (*gltBD*$^+$ *fnr*)	pACYC184	3	NA[c]	2	NA	2	NA
GJ193 (*gltBD fnr*)	pRZ7411-EK209	NA	−	NA	−	NA	−

[a] Derivatives of the indicated strains carrying plasmids pACYC184 (control) or pRZ7411-EK209 (with the *fnr*-EK209 gene) were streaked on glucose-minimal A plates supplemented with 0.75 M NaCl, 1 mM glycine betaine, 20 mM KNO_3, and chloramphenicol and incubated for 42 h at 30°C under ordinarily aerobic, anaerobic, or hyperaerobic conditions (as described in the text). Growth was scored on the following qualitative 4-point scale (in increasing order): − (no growth), ±, +, and ++ (full growth). Expression of *narG*::*lac* (transferred into the appropriate strains from strain RZ8480 by transduction) under the same conditions was determined after the colonies were scraped off the plates and resuspended in minimal A broth for β-galactosidase (β-Gal) assays. Enzyme specific activity values (β-Gal) are given in Miller units (40).
[b] Although GJ972 is a GJ193 derivative and hence carries two additional Mu d1(*lac* Ap) insertions, control experiments with GJ193 have established that neither insertion expresses β-galactosidase to any significant extent (data not shown).
[c] NA, not applicable. GJ973/pACYC184 and GJ193/pRZ7411-EK209 were each used as controls only for *narG*::*lac* expression and for growth, respectively.

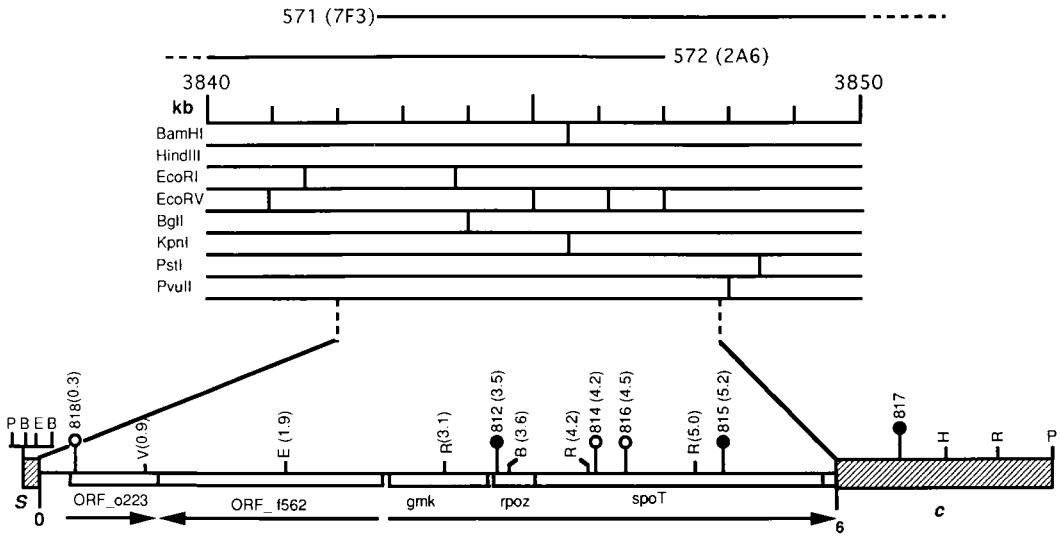

FIG. 3. Physical mapping and Tn*1000*-mutagenesis of plasmid pHYD809. In the lower part of the figure is depicted the structure of the 7.8-kb *Pst*I fragment of pHYD809, consisting of DNA from the Mu *S* and *c* ends (hatched boxes) flanking a 6-kb chromosomal insert (thin line). The Mu *c* end of the fragment is promoter-proximal within the β-lactamase gene of the vector. Restriction enzyme cut sites in the fragment are marked: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RV; and V, *Pvu*II. Circles represent positions of several independent Tn*1000* (γδ) insertions, and the corresponding pHYD plasmid number designations are marked alongside (open circles, Tn*1000* insertions with the γ end to the right and the δ end to the left; solid circles, Tn*1000* insertions in the opposite orientation). Numbers in parentheses refer to kilobase coordinates within the chromosomal insert DNA (left end, 0; right end, 6). Deduced positions of genes and unidentified open reading frames in the insert are indicated as open boxes; arrows mark the direction and extent of transcription (postulated in the case of open reading frames). The upper part of the figure depicts the alignment of the chromosomal insert DNA to the Kohara ordered phage library and the revised physical map of the *E. coli* chromosome in the 3,840- to 3,850-kb region (29, 40). We found an additional *Pvu*II site in the insert (at a position corresponding to 3,843 kb on the *E. coli* chromosome) which is not marked on the Kohara physical map but which is present in the published DNA sequence of this region (4).

was reduced, independently and additively, by increasing pO_2 and by the presence of the negative-dominant EK209 mutation (Table 3). The negative-dominant effect of *fnr*-EK209 in GJ972 also led to abolition of nitrite reductase expression during anaerobic growth, as judged by qualitative tests. Nevertheless, there was no difference at all in the growth of any of these GJ972 derivatives in high-osmolarity medium across the various pO_2 growth conditions (Table 3). These results indicated that the EK209 mutation is recessive to fnr^+ with respect to the osmotolerance phenotype, whereas it is dominant to fnr^+ with respect to regulation of genes in anaerobic respiration.

Identification of recombinant plasmids that restore osmotolerance in GJ193. A library of wild-type *E. coli* chromosomal fragments derived from strain AB1157 and cloned alongside the mini-Mu derivative Mu d5005 (which encodes kanamycin resistance [Kan^r] and carries plasmid pMB9 replication functions) was introduced into the *fnr gltBD* mutant GJ193, with the aid of the in vivo cloning method of Groisman and Casadaban (19). At least six plasmids (with different restriction enzyme cleavage patterns) that conferred an osmotolerance phenotype in GJ193 were identified following selection for Kan^r on glucose-minimal A medium supplemented with 0.7 M NaCl and 1 mM glycine betaine; plasmids from all the osmotolerant clones tested from this selection displayed a characteristic doublet of 1.0- and 0.9-kb bands following *Eco*RV digestion (data not shown), suggesting that the same complementing chromosomal locus had been cloned in all of them. One plasmid derivative (pHYD803) had a 6-kb chromosomal insert, all of which was subcloned on a 7.8-kb *Pst*I fragment (whose two ends were derived from the Mu *c* end and Mu *S* end, respectively, of Mu d5005) into the *Pst*I site of vector pBR329 to generate plasmid pHYD809 (Fig. 3). As expected, pHYD809 transformants of GJ193 were also osmotolerant (Fig. 4).

Multicopy $spoT^+$ as suppressor of osmosensitivity in GJ193. A radiolabelled probe prepared from pHYD809 was shown to hybridize to DNA from λ phage clones 571(7F3) and 572(2A6) in the ordered *E. coli* genomic library of Kohara et al. (29), and subsequent physical mapping experiments also permitted the conclusion that the chromosomal DNA insert on this plasmid corresponded approximately to bp coordinates 8000 to 14000 of the sequence entry ECUW82 (accession no. L10328) in the EMBL DNA sequence database, at 82.2 centisomes on the *E. coli* physical map (data not shown; see Fig. 3) (4, 29, 40, 44). This region includes two unidentified open reading frames (ORF_o223 and ORF_f562) and the complete sequence of the first three genes (*spoR* or *gmk*, *rpoZ*, and *spoT*) of the *spoT* operon (Fig. 3) (4, 14, 46). Results of subcloning experiments (data not shown) indicated that the identified suppressor function was not encoded by ORF_o223, ORF_f562, or *gmk*.

Plasmid pHYD809 was then introduced into strain JP3301 and subjected to Tn*1000* mutagenesis following F-mediated mobilization into GJ971, as described previously (18, 21). Clones which had suffered inactivation by Tn*1000* insertion of the gene encoding the suppressor function on pHYD809 were identified as osmosensitive on medium supplemented with glycine betaine and 0.7 M NaCl (see Fig. 4, where the growth behavior in high-osmolarity medium of two representative Tn*1000*-insertion clones are compared, one in which the suppressor function has been inactivated and the other in which it has been retained). The positions of Tn*1000* insertion in each of four noncomplementing clones (pHYD numbers 812, and 814 to 816), along with those in two other clones in which Tn*1000* insertion had not inactivated the suppressor (pHYD817 and pHYD818) was deduced by physical mapping. The inactivating insertions had occurred in both orientations in either *spoT* itself or the region upstream of *spoT* in the same operon, whereas the control insertions were mapped elsewhere on the plasmid (Fig. 3). These data would indicate that it is the expression of $spoT^+$ (whose product is a ppGpp-3′ pyrophosphohydrolase [ppGppase] and which is postulated to function

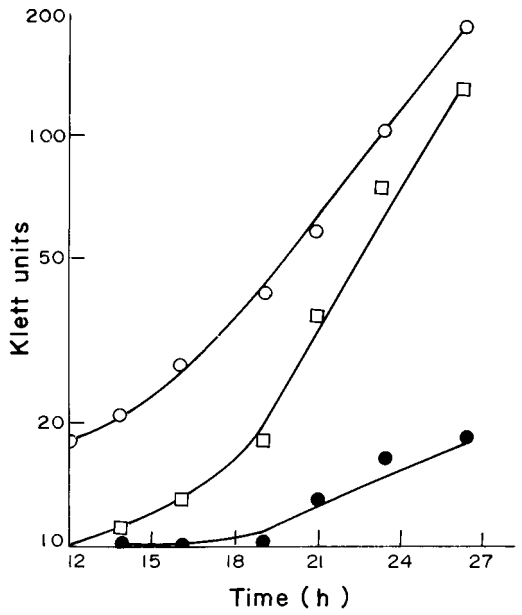

FIG. 4. Suppression of osmosensitivity of *fnr gltBD* strains by multicopy *spoT*$^+$. Derivatives of *fnr gltBD* strains carrying the indicated plasmids were grown to stationary-phase in glucose-minimal A medium and subcultured 1:500 into glucose-minimal A medium supplemented with 0.7 M NaCl and 1 mM glycine betaine. The optical density (Klett units) of each culture was monitored as a function of time of incubation in a shaker water bath at 30°C. Strain symbols (+ and − within parentheses indicate presence and absence, respectively, of multicopy *spoT*$^+$): ○, GJ193/pHYD809 (+); □, GJ971/pHYD817 (+); and ●, GJ971/pHYD814 (−).

also as a ppGpp synthetase [PS-II] during C- or energy-limited growth [15, 25, 53]) from a multicopy plasmid which suppresses osmosensitivity in GJ193.

The approximate extent of the *spoT* gene is from kb coordinates 3.8 to 5.9 in pHYD809 (whose two chromosomal insert DNA ends are taken to represent coordinates 0 and 6 kb, respectively; see Fig. 3); *spoT* would be expressed from the promoters situated at around 2.7 and 3.4 kb (4, 14, 46). This would explain our earlier observation that all the original complementing plasmids carried the *Eco*RV doublet (assuming that all of them express *spoT*), because the corresponding *Eco*RV sites are located at kilobase coordinates 3.13, 4.18, and 5.04 in the insert DNA (Fig. 3).

An earlier characterized minimal-*spoT*$^+$ plasmid pHX41 (53) was also able to confer osmotolerance in the *fnr gltBD* strain GJ920 (data not shown). This result served to exclude the possibility that the upstream *rpoZ* gene is necessary for the suppressor phenotype.

Growth rescue by multicopy *spoT*$^+$ of *gltBD* mutants on low [NH_4^+]. We tested whether multicopy *spoT*$^+$ conferred osmotolerance by suppressing or bypassing the requirement of either of the mutations *fnr* or *gltBD* in GJ193. *fnr*-dependent phenotypes (anaerobic regulation of nitrite reductase or of *narG*::*lac* expression) were unaffected by the introduction of pHYD809 into appropriate *fnr* mutant strains (data not shown). The plasmid, however, complemented the *gltBD* mutation in GJ193 for growth on plates containing either 0.4 mM NH_4^+ or 0.2% L-alanine, L-glutamate, or L-proline as the N source (Table 4). With the use of *gltBD* derivatives of strains such as CF1648 and JM101, we could also conclude that suppression of *gltBD* by pHYD809 was independent of the genotypes at *fnr* and at *relA* (whose product is the other major player in intracellular ppGpp metabolism [6]) (data not shown). Furthermore, introduction of pHYD809 had no appreciable effect on growth rates of *gltBD*$^+$ strains under these conditions (data not shown). In all cases tested, the *spoT*::Tn*1000* insertion mutations abolished growth complementation of the *gltBD* mutants (data not shown).

pHYD809 transformants of GJ193 were not complemented for growth on Ntr-regulated N sources such as L-arginine and L-ornithine (Table 4), suggesting that (i) *spoT*-mediated suppression does not extend to functions that are regulated by *gltF*, the gene downstream of *gltBD* in the same operon (7, 8), and (ii) osmosensitivity associated with the *gltBD238*::*lac* insertion is itself not due to a polar effect on the expression of *gltF*. Plasmid pHYD809, as well as the minimal *spoT*$^+$ plasmid pHX41, was able to complement even a Δ*gltBDF fnr* strain (GJ946) for both osmotolerance and growth on low [NH_4^+] (data not shown), indicating that the observed suppression is indeed a bypassing of the need for GOGAT in low-[NH_4^+] growth medium.

GDH and GOGAT activity in pHYD809 derivatives of *gltB* mutants. The most likely means by which this bypass could occur is through an activation of the GDH pathway such that it is able to carry out the synthesis of L-glutamate even under low-[NH_4^+] conditions. In support of a role for GDH in the phenotypic suppression, we found that GDH specific activity in cell extracts prepared from the pHYD809 transformant of strain GJ946 was approximately threefold higher than that in extracts from the control pBR329 transformant (Table 2). GJ946/pHYD809 also exhibited a low level of GOGAT activity (even though it is Δ*gltBD*) (Table 2). We believe, however, that the latter is an artifact of increased GDH activity in these cell extracts (in conjunction with the [glutaminase-mediated] release of low levels of NH_4^+ from the glutamine added during the GOGAT assay). Cell extracts of GJ946/pHYD809 also displayed a higher endogenous NADPH oxidation activity than did the control (76 versus 31 mU/mg of protein, respectively), and perhaps for the same reason. Nevertheless, the alternative possibility that GDH in GJ946/pHYD809 has acquired an ad-

TABLE 4. Alteration of N-source utilization ability of an *gltBD* mutant (GJ193) by pHYD809 and by nature of C source[a]

Strain	Growth on:															
	3 mM NH_4^+		0.4 mM NH_4^+		Glycine		L-Alanine		L-Glutamate		L-Arginine		L-Proline		L-Ornithine	
	D[b]	G	D	G	D	G	D	G	D	G	D	G	D	G	D	G
MC4100 (wild-type)	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
GJ193	++	++	−	+	−	−	−	±	±	±	−	−	−	+	−	−
GJ193/pHYD809	++	++	++	++	−	++	+	++	+	++	−	−	+	++	−	−

[a] Strains were streaked on W salts medium supplemented with the indicated N and C sources. After incubation for 42 h at 30°C, growth was scored on the four-point scale described in Table 3, footnote *a*.

[b] D, glucose (0.4%), and G, glycerol (0.4%), were the two alternative C sources used in the experiments.

ditional catalytic activity following some change in posttranslational modification (34) has not been excluded in our studies.

Effect of C source on growth of *gltBD* mutants on low [NH_4^+]. Helling (24) has demonstrated that GDH-catalyzed N assimilation is important during energy-limited growth of *E. coli*. Energy limitation leads to an increase in intracellular ppGpp mediated by the (activation of PS-II and/or inhibition of ppGppase) activity of the *spoT* gene product (6, 53), and hence our observations appeared to be mechanistically linked to those of Helling (24). We proceeded to examine the effect of using a C source poorer than glucose (such as maltose, glycerol, or succinate) on N assimilation by *gltBD* strains (with and without pHYD809). The slower growth rate supported by the use of these alternative C sources was expected to be associated with an increase in intracellular ppGpp (6), and measurements to this effect had in fact been made in the case of maltose (27).

Remarkably, we discovered that the view traditionally held (43), that GOGAT is indispensable for growth on medium with <1 mM NH_4^+, is valid only for glucose-supplemented culture media. With the other three C sources tested, the *gltBD* strains (that were haploid for *spoT*$^+$) could grow moderately well on an intermediate concentration (0.4 mM) of NH_4^+ (see Table 4 for representative data obtained for strain GJ193 with glycerol). The effects of a poor C source (glycerol or succinate) and of the *spoT*$^+$ plasmid pHYD809 on utilization of some N sources appeared to be additive or synergistic, as seen from the data for growth on glycine, L-alanine, L-glutamate, and L-proline (Table 4). On the other hand, growth on *gltF*-regulated N sources such as L-arginine and L-ornithine was not restored under any of these conditions (Table 4). Once again, the observed effects were independent of the *fnr* and *relA* genotypes of the strains (data not shown).

DISCUSSION

Synergism between *gltBD* and *fnr* mutations for osmosensitivity. Csonka et al. (13) had earlier shown that *gltBD* mutants are osmosensitive in low-[NH_4^+] media, that is, under conditions when L-glutamate synthesis is compromised. The results described in this paper indicate that (i) a mutant (GJ193) isolated as osmosensitive on high-[NH_4^+] medium harbors mutations in *gltBD* and *fnr*; (ii) replacement of NH_4^+ with L-aspartate or L-asparagine abolishes its osmosensitivity; and (iii) a multicopy plasmid which suppresses the *gltBD* defect in the mutant also confers osmotolerance. These data implicate a defect in N assimilation as the basis for the osmosensitive phenotype of GJ193 and provide additional evidence that increased L-glutamate synthesis and accumulation are important in *E. coli* osmoregulation (2, 13, 37). Implied in this conclusion is the existence of a mechanism at high osmolarity for readjustment of the set point in feedback control of L-glutamate synthesis via both the GOGAT and GDH pathways (7, 43), but the details of this mechanism are not known.

Our results on osmosensitivity of *gltBD* mutants differ from those of Csonka et al. (13) in that they have been obtained with media with high [NH_4^+]. The simplest explanation for our data would therefore be that GDH-catalyzed N assimilation is markedly deficient in *fnr* strains grown at high osmolarity even in ammonia-rich medium. The moderate reduction in growth rate of GJ921 (*fnr*$^+$ *gltBD*) (Fig. 1) observed under these conditions would suggest that even with functional FNR, N assimilation through the GDH pathway may be limiting for growth at high osmolarity. In the absence of a *gltBD* defect, there is no additivity of mutations in *fnr* with other osmosensitive mutations such as *ots*, *proU*, and *proP* (data not shown).

The mechanism by which *fnr* mutations might affect GDH-mediated N assimilation is not clear, but the role of FNR as a regulator protein in mediating this function is suggested by the observation that FNR-EK209 (which is affected in the DNA-binding domain) is itself not competent for osmotolerance. Our results also indicate that the specific activity of GDH is not altered in *fnr* mutants (Table 2); it is possible that NH_4^+ uptake, or the concentration of small-molecular-weight substances that modulate the activity of this enzyme in vivo, is affected by FNR.

Monomeric FNR in osmoregulation. With the combined use of hyperaerobic growth conditions and the *trans*-dominant EK209 mutation in FNR, we were able to reduce the concentration of dimeric functional FNR species in cultures to a level that supported $<3\%$ of the induced level of expression of a canonical FNR-regulated gene, and yet the osmotolerance phenotype was completely unaffected by these perturbations. Once again, the simplest interpretation of these data would be that monomeric FNR is functional aerobically in osmoregulation whereas dimeric FNR is required for in vivo activation of at least some of the genes in anaerobic respiration. However, the model is still somewhat speculative, primarily because the mechanism of action of FNR in osmoregulation and the presumed target genes that subserve this function are as yet unknown. It is also not clear whether dimeric FNR can participate in osmoregulation.

ppGpp metabolism and *gltBD* suppression. The identification in this work of *spoT*$^+$ as a multicopy suppressor of *gltBD* implicates ppGpp as a potential regulatory molecule in the GDH-catalyzed pathway of NH_4^+ assimilation. Our data from the enzyme assays (Table 2) indicate that the multicopy *spoT*$^+$ derivative exhibits an elevated GDH activity which could account for the observed suppression.

Since SpoT has two opposing activities that mediate both ppGpp synthesis and degradation (15, 25, 53), it is at present uncertain whether GDH activation in these multicopy *spoT*$^+$ derivatives is effected by increased or decreased levels of ppGpp. Overexpression of *spoT*$^+$ in cultures growing exponentially in rich medium is associated with a more marked increase in ppGpp-degradative activity than in PS-II activity (25, 46). On the other hand, Xiao et al. (53) have shown that the multicopy minimal *spoT*$^+$ plasmid pHX41 restores ppGpp accumulation in a Δ*relA* Δ*spoT* strain subjected to C-source limitation, indicating that the ratio of PS-II to ppGppase activity of SpoT is increased under these conditions.

Although intracellular ppGpp measurements are necessary before one can unequivocally establish the mechanism by which the presence of multicopy *spoT*$^+$ results in *gltBD* suppression, the following arguments lead us to suggest that it may be an increase in ppGpp which is responsible for the phenomenon. (i) We find that the use of poor C sources partially alleviates the low-[NH_4^+] growth defect associated with *gltBD* mutations even in haploid *spoT*$^+$ strains (Table 4). It is well established that C limitation is associated with a SpoT-mediated increase in intracellular ppGpp (6, 27, 53). (ii) In many instances, plasmid pHYD809 appears to accentuate the effect of a poor C source in achieving *gltBD* suppression (Table 4), and as mentioned above, other workers have shown that multicopy *spoT*$^+$ leads to an increase in intracellular ppGpp under similar conditions (53). (iii) Finally, osmotic stress is associated with increased ppGpp, which is also apparently mediated by a RelA-independent, SpoT-dependent mechanism(s) (22, 23). We, therefore, consider it likely that in cells with pHYD809, the ratio of the PS-II to ppGppase activity of SpoT is elevated under the conditions used for observing *gltBD* suppression.

Implications of GDH activation during C-limited growth. Irrespective of the exact mechanism by which C-source limitation leads to GDH activation, this finding itself bears relevance to our understanding of the role of GDH in *E. coli* survival and growth in situ, that is in the lumen of the mammalian large intestine and in soils, sediments, or water into which it is voided (47). In these environs, the estimated generation time of *E. coli* is between 12 and 24 h, with limitation of (and competition from other flora for) both N and C sources (28). In its extraintestinal habitat, there is also the possibility of its experiencing desiccation stress.

In strains that are *gltBD*$^+$, *gdhA* mutations confer no overt phenotype (43), thereby raising a question regarding the role of GDH in *E. coli*. Helling (24) has recently shown that GDH-catalyzed N assimilation, being less energy intensive than that catalyzed by GOGAT, provides a competitive growth advantage to cultures specifically grown in energy-limiting ammonia-rich medium. On the basis of the present results, we can now extend this conclusion to suggest that *E. coli* can efficiently assimilate NH_4^+ via the GDH pathway even when it is osmotically stressed or is simultaneously N and C starved. Thus, the ecological milieu of *E. coli* appears to fulfill all the conditions that are required for, and in turn necessitate, the optimal functional activity of GDH as a catalyst of the second pathway for NH_4^+ assimilation in the organism.

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