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 NH 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 gltD, 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 cascaded 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 P1Kc was from our laboratory stocks. λp1(209) phage was obtained from M. J. Casadaban. The phages λK370, λ1096, and λ1105, used as vectors for the transposition of Tn10, Tn50Dtet, and Tn50dKan, respectively, were obtained from N. Kleckner. A
TABLE 1. *E. coli* K-12 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157 (Mu cts)*</td>
<td>thr-1 ara-14 leuB6 Δ(qpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpoS38 rpsL31 dkgK51 xyl-5 mil-1 argE3 thi-1 (Mu cts)</td>
<td>R. Jayaraman</td>
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<tr>
<td>CAG12153</td>
<td>zha-6::Tn10</td>
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<tr>
<td>CF1648</td>
<td>Wild-type</td>
<td>Same as MG1655 (53)</td>
</tr>
<tr>
<td>CSH57</td>
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<td>JM101</td>
<td>supE thi Δ(qpt-lac)5 F+ [trnat36 proAB lacI1lac2ΔM15]</td>
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<tr>
<td>JP2144</td>
<td>his-29(Am) rpsL4906l(Am) ih-1 tyr606</td>
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</tr>
<tr>
<td>JP2769*</td>
<td>his-29(Am) ih-1 zy-352::Tn10</td>
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</tr>
<tr>
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<tr>
<td>recA56 [Δ(pheA-lac)]</td>
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</tr>
<tr>
<td>JRG661-b</td>
<td>fnr-8</td>
<td>48</td>
</tr>
<tr>
<td>KL14*</td>
<td>Hfr(HO68) thi-1 relA spoT1 fnr-267</td>
<td>B. J. Bachmann</td>
</tr>
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<td>MAL103</td>
<td>araB::Mu cts araD139 Δ(gpt-lac)5 rpsL [Mu d1(dac Ap)]</td>
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<tr>
<td>MC4100</td>
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<td>PA340</td>
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<td>RZ48408</td>
<td>lacZΔ145 Δ(hfr-1)(Sm^3 Sp^) narG234::Mu d1734(lac Kan)</td>
<td>32</td>
</tr>
<tr>
<td>GJ193*</td>
<td>MC4100 fnr-266 gadBD238::Mu d1(lac Ap)</td>
<td>This study</td>
</tr>
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<td>GJ309</td>
<td>GJ193 fnr-1 zda-901::Tn10</td>
<td>This study</td>
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<td>This study</td>
</tr>
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<td>GJ910</td>
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<tr>
<td>GJ912</td>
<td>GJ193 zda-901::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>GJ913</td>
<td>GJ396 gadBD238::Mu d1(lac Ap) zha-901::Tn10DelKan</td>
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</tr>
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<td>GJ920</td>
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</tr>
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<td>MC4100 zha-901::Tn10DelKan gadBD238::lac</td>
<td>This study</td>
</tr>
<tr>
<td>GJ930</td>
<td>GJ921 fnr-8 zyi-352::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>GJ946</td>
<td>GJ396 ΔglyBDF500 zha-6::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>GJ959</td>
<td>GJ921 Δ(hfr-1)(Sm^3 Sp^)</td>
<td>This study</td>
</tr>
<tr>
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<td>MC4100 Δ(hfr-1)(Sm^3 Sp^)</td>
<td>This study</td>
</tr>
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<td>This study</td>
</tr>
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<td>GJ193 recA srl::Tn10</td>
<td>This study</td>
</tr>
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<td>GJ972</td>
<td>GJ311 narG234::Mu d1734(lac Kan)</td>
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<td>GJ973</td>
<td>GJ396 narG234::Mu d1734(lac Kan)</td>
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<tr>
<td>GJ1235</td>
<td>MC4100 argR203</td>
<td>G. Uma Prasad</td>
</tr>
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</table>

* 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).

* The position of the Mu insertion in AB1157 (Mu cts) is not known. The presence of the rpoS38 mutation in AB1157 was identified for the first time in this laboratory (36).

* The insertion previously designated zyi-2::Tn10 (9) has now been redesignated as zyi-352::Tn10 (42a).

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

* fnr-266 and gadBD238::Mu d1(lac Ap) are redesignations, respectively, of the oxaA and ooxB mutations in GJ193, described in the text. GJ193 also carries an additional Mu d1(lac Ap) insertion at an undefined site, as indicated by the fact that gadBD-3 transductants of GJ193 (such as GJ910) continue to be Amp+ and temperature sensitive.

* GJ396 was obtained as a spontaneous Tet- mutant of GJ395.

* GJ971 was constructed by transduction of recA linked to srl::Tn10 from strain GJ216 (26) into GJ193.

lysat 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 (Ts) derivative carrying the plasmid pEG5005, as described previously (19).

The plasmid vectors pACYC184 (40), pBR322 (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+ plasmid vector [53]), and pKZ711/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 BamHI-HindIII 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 auxotropic 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) NH4Cl at the desired concentration or any of the indicated amino acids at 0.2%. Unless otherwise specified, antibiotics were used in 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 p-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 p-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 107 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 growth 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 pHs 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 (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, at least 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 KNO3 as an inducer) and also by the test of methylene blue reduction in the desiccator. Hypo- and hyperaerobic conditions relate to growth on plates in a sealed vacuum desiccator (as above) that had been flushed and filled with O2 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 KNO3 (32).

Transposon techniques. Random transpositions of Tn10, Tn10/dKan, or Tn10/dKan derivatives of MC4100 obtained in the reverse transduction cross were not osmosensitive, indicating that the osmA mutation was necessary but not sufficient for exhibition of this phenotype. The MC4100 osmA transductants also continued to be Amp– and Mu sensitive, indicating that the concerned mutation was not a Mu d1(lac Ap) insertion.

Random transpositional insertions of Tn10dKan or of Tn10dKan were then separately generated into the chromosome of GJ193 (an osmA derivative of MC4100), and P1 lysates prepared on pools of such clones were used again to transduce GJ193 simultaneously to Tet– or Kan– (as appropriate) and to osmotolerance. In this manner, one Tn10dKan insertion allele (designated zha-900) was identified that was almost 100% linked to one another and that were each in turn 80% linked to a gene (designated osB) in GJ396 that by itself conferred osmotolerance upon introduction into GJ193. A second mutant strain with simultaneous selection for transposon marker activity was obtained and physically mapped as described by Gucer (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 (16), 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-32P]dATP (purchased from BRL, Department of Atomic Energy, Bombay, India), and hybridization to blotted DNA on 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 P1kc transduction (16) were as described previously. Spontaneous Tet‘ derivatives of Tet‘ strains were selected by the protocol of Maloy and Nunn (35). The method of Komeda and Iino (38) was used to convert the Mu d1(lac Ap) lysogen GJ193 into a temperature-resistant, Amp‘ derivative, GJ390, which now carries a KpnI(209) prohage insertion at the mutant locus.

Results

Osmosensitivity of GJ193 and identification of two mutant loci, osmA and osmB. 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 osmA 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, osmA and osmB: Mu d1(lac Ap), that are together necessary for osmosensitivity. A P1kc lysate prepared on a population of Tn10-mutagenized MC4100 clones was used to transduce GJ193 simultaneously to Tet‘ and osmotolerance. Two of the colonies so obtained had Tet‘ insertions (designated zda-900: Tn10 and zha-901: Tn10 based on the mapping data below) 99 and 50% cotransducible, respectively, with the MC4100 locus designated osmA. Whereas osmB transductants of GJ193 were significantly osmotolerant, osmA derivatives of MC4100 obtained in the reverse transduction cross were not osmosensitive, indicating that the osmA mutation was necessary but not sufficient for exhibition of this phenotype. The MC4100 osmA transductants also continued to be Amp‘ and Mu sensitive, indicating that the concerned mutation was not a Mu d1(lac Ap) insertion.

Random transpositional insertions of Tn10dKan or of Tn10dKan were then separately generated into the chromosome of GJ396 (an osmA derivative of MC4100), and P1 lysates prepared on pools of such clones were used again to transduce GJ193 simultaneously to Tet‘ or Kan‘ (as appropriate) and to osmotolerance. In this manner, one Tn10dKan insertion allele (designated zha-900: Tn10dKan and zha-901: Tn10dKan, respectively) were identified that were almost 100% linked to one another and that were each in turn 80% linked to a gene (designated osmB) in GJ396 that by itself conferred osmotolerance upon introduction into GJ193. A pair of isogenic Tn10dKan derivatives of GJ193 carrying either the wild-type or the mutant osmB allele at this locus were designated GJ910 and GJ912, respectively.

When GJ912 was used as the donor in transduction into the osmA mutant GJ396, approximately 5% of the Kan‘ colonies had now become as osmosensitive as the original mutant, GJ193 (data not shown). These colonies (one representative designated GJ913) were also Amp‘, Mu immune, and temperature sensitive for growth and remained Lac‘, permitting the following conclusions: (i) the mutation at osmB 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‘ insertion with osmB‘ (80%) and with osmA: 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, osmA and osmB, in GJ193 are necessary and sufficient for exhibition of the osmosensitive phenotype.

The availability of the linked transposon markers enabled
the construction, by P1kc transduction, of defined isogenic
ossA (GJ396), ossB (GJ921), and ossA ossB (GJ920) deriva-
tives of the wild-type strain MC4100. [The ossB:Mu d1(lac
Ap) allele used in these constructions had earlier been stabi-
lized by the method of Komeda and Iino (30).] Tests of osmo-
tolerance 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::Tn10-tyrR-(ossA-zda-900::Tn10)-zda-901::Tn10 and
argG-ossB-(zha-900::Tn10dTet-zha-901::Tn10dKan)-zha-6::
Tn10-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 NH4+ assimilation and l-
glutamate biosynthesis, particularly when the availability of
NH4+ in the medium is low (43). The following lines of evi-
dence indicate that ossB is an insertion in gltBD.

(i) Growth of gltBD mutants is either abolished or substi-
tially reduced on W salts medium supplemented with <1 mM
NH4+ 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 negli-
gible GOGAT activity and normal GDH activity (the alterna-
tive enzyme for glutamate biosynthesis at high [NH4+] )
compared with the levels in MC4100 (Table 2).

(iii) Strain GJ946, which was constructed by introducing a
known ΔgltD5050 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 by-
pass 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-
[NH4+] medium. We therefore conclude that the ossB muta-
TABLE 2. GDH and GOGAT activities in *ossA (fnr)* and *ossB (gltBD)* derivatives

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Sp. act. (mU/mg of protein)</th>
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<td></td>
<td></td>
<td>GDH</td>
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<tr>
<td>MC4100</td>
<td>Wild-type</td>
<td>1.090</td>
</tr>
<tr>
<td>GJ396</td>
<td><em>fnr-266</em></td>
<td>1.140</td>
</tr>
<tr>
<td>GJ920</td>
<td><em>fnr-266</em> <em>gltBD238</em></td>
<td>1.370</td>
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<tr>
<td>GJ940/pBR329</td>
<td><em>ΔgltBD fnr-266</em> vector</td>
<td>1.250</td>
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<td>GJ940/pHYD809</td>
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<td>3.170</td>
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</table>

* Enzyme specific activities were determined in cell extracts of cultures grown to mid-log phase at 37°C in 0.4% glucose–2 mM NH₄⁺–W salts medium with antibiotic supplementation as appropriate.

* ND, not determined.

*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 *Δfnr::Ω* mutations from JRG861-b and RZ8480, respectively, into the *ossA* *gltBD* strain GJ912, were osmosensitive (data not shown). Likewise, the *ΔgltBD* *Δfnr* 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 *Δfnr::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) transfectants of both GJ920 (*ossA* *gltBD*) and GJ921 (*ossA* *ΔgltBD*) 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 P**₂**O**₇** 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 (GJ913), 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>
<thead>
<tr>
<th>Strain derivative (chromosomal genotype)</th>
<th>Plasmid</th>
<th>Anoxic</th>
<th>Ordinarily aerobic</th>
<th>Hyperaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>β-Gal</td>
<td>Growth</td>
<td>β-Gal</td>
</tr>
<tr>
<td>GJ972 (gltBD <em>fnr</em>⁺)</td>
<td>pACYC184</td>
<td>310</td>
<td>+ +</td>
<td>72</td>
</tr>
<tr>
<td>GJ972 (gltBD <em>fnr</em>⁻)</td>
<td>pRZ7411-EK209</td>
<td>28</td>
<td>+ +</td>
<td>18</td>
</tr>
<tr>
<td>GJ973 (gltBD  * fnr)</td>
<td>pACYC184</td>
<td>3</td>
<td>NA*</td>
<td>2</td>
</tr>
<tr>
<td>GJ913 (gltBD <em>fnr</em>)</td>
<td>pRZ7411-EK209</td>
<td>NA</td>
<td>–</td>
<td>NA</td>
</tr>
</tbody>
</table>

* 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 KNO₃, 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).

* Although GJ972 is a GJ193 derivative and hence carries two additional Mu *d(lac) Ap* insertions, control experiments with GJ193 have established that neither insertion expresses β-galactosidase to any significant extent (data not shown).

* NA, not applicable. GJ973/pACYC184 and GJ193/pRZ7411-EK209 were each used as controls only for *narG::lac* expression and for growth, respectively.
was reduced, independently and additively, by increasing pO₂ 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 pO₂ 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 osmotosensitivity 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⁺] 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⁺ 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 EcoRV digestion (data not shown), suggesting that the same complementing chromosomal loci 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 PstI fragment (whose two ends were derived from the Mu c end and Mu S end, respectively, of Mu d5005) into the PstI 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, spoZ, 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 Tn1000 mutagenesis following F-mediated mobilization into GJ971, as described previously (18, 21). Clones which had suffered inactivation by Tn1000 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 Tn1000-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 Tn1000 insertion in each of four noncomplementing clones (pHYD numbers 812, and 814 to 816), along with those in two other clones in which Tn1000 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‘-pyrophosphorylase [ppGppase] and which is postulated to function...
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 EcoRV doublet (assuming that all of them express spoT), because the corresponding EcoRV 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 for gltBD strain GJ920 (data not shown). This result served to exclude the possibility that the upstream ppoZ gene is necessary for the suppressor phenotype.

Growth rescue by multicopy spoT of gltBD mutants on low [NH4⁺]. We tested whether multicopy spoT conferred osmotolerance by suppressing or bypassing the requirement of either of the mutations for or gltBD in GJ193. for-dependent phenotypes (anaerobic regulation of nitrite reductase or of narG::lac expression) were unaffected by the introduction of pHYD809 into appropriate for mutant strains (data not shown).

The plasmid, however, complemented the gltBD mutation in GJ193 for growth on plates containing either 0.4 mM NH4⁺ 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 for 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::Tn1000 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 ΔgltBD for strain (GJ946) for both osmotolerance and growth on low [NH4⁺] (data not shown), indicating that the observed suppression is indeed a bypassing of the need for GOGAT in low-[NH4⁺] 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-[NH4⁺] 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 ΔgltBD) (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 NH4⁺ 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 mM/mg of protein, respectively), and perhaps for the same reason. Nevertheless, the alternative possibility that GDH in GJ946/pHYD809 has acquired an ad-

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>3 mM NH₄⁺</th>
<th>0.4 mM NH₄⁺</th>
<th>Glycine</th>
<th>l-Alanine</th>
<th>l-Glutamate</th>
<th>l-Arginine</th>
<th>l-Proline</th>
<th>l-Ornithine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D⁺  G</td>
<td>D  G</td>
<td>D  G</td>
<td>D  G</td>
<td>D  G</td>
<td>D  G</td>
<td>D  G</td>
<td>D  G</td>
</tr>
<tr>
<td>MC4100 (wild-type)</td>
<td>++ + + + +</td>
<td>++ + + + +</td>
<td>++ + + + +</td>
<td>++ + + + +</td>
<td>++ + + + + +</td>
<td>++ + + + + +</td>
<td>++ + + + + +</td>
<td></td>
</tr>
<tr>
<td>GJ93</td>
<td>++ + + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td></td>
</tr>
<tr>
<td>GJ193/pHYD809</td>
<td>++ + + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td></td>
</tr>
</tbody>
</table>

* 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.

* 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 dispensable 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 glyR-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$_4^+$ by 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 osmolality 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 osmolality 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 osmolality. In the absence of a gltBD defect, there is no additive 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 ΔrelA 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*, *gldA* 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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REFERENCES


4114  SAROJA AND GOWRISHANKAR  J. BACTERIOL.


