Salmonella enterica can use ethanolamine as a sole source of carbon and nitrogen. Ethanolamine is degraded in two enzymatic steps. (i) The EutABC enzyme catalyzes cobalamin requirement for growth on ethanolamine in a low pH. Evidence is presented that protonated ethanolamine (EthH) does not enter cells, while uncharged ethanolamine (Eth0) diffuses freely across the membrane. The external concentration of Eth0 varies with the pH (pK = 9.5). At pH 7.0, the standard ethanolamine concentration (41 mM) provides enough EthH for an influx rate that can support growth with or without EutH. When a lowered pH and/or ethanolamine concentration reduced the EthH concentration below 25 μM, EutH was needed to facilitate diffusion. EutH+ cells grew normally at EthH concentrations above 3 μM, close to the Km (9 μM) of the first degradative enzyme, ethanolamine ammonia lyase. It is suggested that EutH facilitates diffusion of EthH. As predicted for a transporter, EutH contributed to the toxicity of ethanolamine seen under some conditions; furthermore, fusion of EutH to fluorescent Yang protein provided evidence that EutH is a membrane protein.

Materials and Methods

Strains and crosses. All of the strains used in this study (Table 1) are derivatives of S. enterica serovar Typhimurium strain LT2. Transductional crosses were mediated by a high-frequency transducing mutant of phage P22 (HT105, int) (21). The procedures used for transduction crosses and phage lysate preparation were previously described (17).

Media and growth conditions. The rich medium used was nutrient broth (Difco) with 0.1 mM NaCl. The standard minimal medium used was NCE (2) with the indicated carbon source or nitrogen-free NCE medium (NCN) with the indicated carbon and nitrogen sources. The minimal medium used for growth rate determinations contained 5 mM KH2PO4, 5 mM NaNH4HPO4, and 1 mM MgSO4 and was buffered at various pHs with 50 mM morpholineethanesulfonic acid (MES; pHs 5.5 and 6.0), morpholinepropanesulfonic acid (MOPS; pHs 6.5, 7.0, and 7.5), or HEPES (pH 8.0). Ethanolamine hydrochloride and sodium succinate (Sigma) served as carbon sources at the indicated concentrations. In the construction of a eutE mutation (below), glycerol (20 mM; Mallineckrodt) was used as a carbon source in NCN medium with ethanolamine as a nitrogen source (2). Cyanocobalamin (Sigma) was used as a supplement at 150 mM. All minimal liquid media contained biotin, Ca–d-pantothenic acid, nicotinamide, and pyridoxine HCl at 4 x 10^-5 M (wt/vol); thiamine and riboflavin at 2 x 10^-5 M; and trace metals as previously described (13). Rich medium was solidified by agar (1.5%; EM Science), and minimal medium was solidified by Noble agar (US Biological). Liquid cultures were lightly aerated by shaking standing culture tubes at 240 rpm. For measuring growth in liquid media, 3-ml nutrient broth cultures were started from single colonies and grown overnight. Cells were then pelleted, washed in pH 7.0 minimal medium, resuspended in the same medium, and used (35 μl) to inoculate 6 ml of growth medium. Growth was monitored in
three parallel cultures by following the optical density at 650 nm (OD 650) of samples against a standard curve prepared with the culture grown to mid-log phase.

Construction of eutE and eutH mutations. Deletion mutations were constructed by linear transformation as described by Murphy et al. (11) and did not disturb the reading frame of the deleted gene. Final constructions were transduced by P22-mediated crosses into a wild-type LT2 background and verified by PCR and sequencing.

To make a eutE deletion, a chloramphenicol resistance (cat) cassette was amplified with primers P1 (5'ATGAAATACAGATATTTGAACAGGTGGTACGACACCAGCATGCGAGGAATAGGAACTTCACGGGGAGAGCCTGAGCAAA). This fragment was amplified using primers P3 (5'TACATCATGATGTTCTCACCAAACACCCCCCAAAACC) and P4 (5'TCTTGATATCG).

The 5' end of P1 (40 bp) has a sequence identical to the distal end of the cat gene. The 5' end of P2 (42 bp) is the reverse complement of the last 42 bp of eutE and is followed by the reverse complement of an FRT site. The 3' end is identical to the distal end of the cat cassette. The linear fragment produced by PCR amplification of cat was used to transform strain TT2236 to CamR. Plasmid pCP20, encoding FRT recombinase, was introduced. Recombination between FRT sites removed the CamR cassette, leaving a wild-type deletion; the mutant gene produces a small peptide with the first 15 and the last 13 amino acids of the EutE sequence plus an in-frame extension at the N-terminus.

A eutH deletion was constructed by replacing a eutH::Tn10 insertion with a short in-frame sequence unrelated to eutH. A short in-frame sequence unrelated to eutH was amplified with primers P1 (5'ATGGGAAATACAGATATTTGAACAGGTGGTACGACACCAGCATGCGAGGAATAGGAACTTCACGGGGAGAGCCTGAGCAAA) and P2 (5'TTTATACATGCGAAACCGA).

The 5' end of P3 is identical to the first 40 bp of eutH, and the 3' end is complementary to the 5' end of the linker. The 5' end of P4 is reverse complementary to the last 40 bp of eutH, and the 3' end is a reverse complement of the 5' end of P4.

A double mutant carrying both the eutE and eutH in-frame deletions was constructed by a series of P22-mediated transductions. A phage lysate grown on a double-insertion strain (eutH::Tn10, eutE::Tn10) was used to transform strain TT2236 to CamR. Plasmid pCP20, encoding FRT recombinase, was introduced. Recombination between FRT sites removed the CamR cassette, leaving a wild-type deletion; the mutant gene produces a small peptide with the first 15 and the last 13 amino acids of the EutE sequence plus an in-frame extension at the N-terminus.

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The gene for yellow fluorescent protein (yfp) from the Clontech pEYEP vector was fused to the distal end of the eutH gene by two successive linear transformations that introduced the yfp coding region into the eut operon without causing a polar effect on transcription. The first transformation introduced a cassette that includes the sacB and kan genes between the chromosomal eutH and eutA genes (8, 10). The sacB gene confers sucrose sensitivity, and the kan gene provides resistance to kanamycin. The cassette was amplified with primers EutHsacF (CGCGCGCCATGGGAAACCGAAAGGCGAGGAACATCGT GAGTGCGGCGGCTGCTAGAACTAGTG) and EutH sacR (GTGCCGATATC GACACCAGCATGCGAGGAATAGGAACTTCACGGGGAGAGCCTGAGCAAA).

The SacKan cassette was then replaced with the yfp coding region with selec-
tion for sucrose resistance. For this, we used a PCR fragment created by amplifying the yfp gene from the pEYFP vector with primers eutHeyfpF (GACGCGGCCAGGTGAAAACCGAAGCGGAGCGCATTGGGAAGTTGGCGGAGGAAGC) and eutHeyfpR (GGATATCGATACCAGGCTCAGTGGAGGTGGTCTCA CGATTGGCCTCCGCCTTTGTACAGGTCCGTCCATG).

Sucrose-resistant transformants had lost Kn' and carried a eutH-yfp gene fusion that was verified by sequencing (TT24899).

Cell swelling assays. The method of Heller et al. (5) monitors the shrinkage and reswelling of cells in response to a concentrated solution of a test compound. Ethanolamine (shrinkage), followed by influx of ethanolamine and water (reswelling), was followed over a 15-min time course. Cells were pregrown overnight in NCE medium with 0.2% ethanolamine hydrochloride, 200 mM cyanocobalamin (B12), and 1% sodium succinate. Overnight cultures were diluted 1:200 into 1 liter of the same culture medium and shaken in 2-liter Kimax flasks at 37°C until the culture reached an OD600 of approximately 1.0. Cells were pelleted by centrifugation, washed twice with 50 mM MOPS (pH 7), and resuspended in ethanolamine buffer and monitoring the OD600 every 6 s for 15 min with a Beckman Instruments DU series 600 spectrophotometer.

RESULTS

Ethanolamine crosses the cell membrane in a pH-dependent manner. The cell swelling assay (5) measures solute entry into cells indirectly through changes in the turbidity of a cell suspension that result from plasmolysis (shrinkage) and deplasmolysis (reswelling) of cells after solute addition. Shrinkage results from water loss caused by exposure of cells to an external hypertonic solution, and reswelling results from entry of the solute accompanied by water (1). These assays (Fig. 3) show that, at pH 7, ethanolamine (250 mM) caused shrinkage but no reswelling, suggesting no entry of the solute. At a higher pH, the amount of shrinkage decreased (smaller ΔOD600 between the baseline and the ethanolamine curve at time zero), and the rate of reswelling increased (slope of OD600 curve returning to the baseline). The highest reswelling rate was at pH 10.5. At this pH, ethanolamine is mostly unprotonated since the pH for protonation is 9.5. A eutH mutation had no effect on this assay (data not shown), suggesting that the massive ethanolamine influx required to reswell cells is not dependent on this channel. These data indicate that the charged species, which predominates at low pH, does not enter cells at a rate that can be seen in this assay. These results suggested that EutH may not be needed at pH values sufficiently high to allow unaided diffusion. More detailed implications of these results are discussed later.

The ethanolamine growth phenotype of a eutH mutation is pH dependent. The swelling assay suggested that uncharged ethanolamine enters cells by diffusion at a rate that might be sufficient to support growth at standard pH without a specific transporter. That is, a eutH mutant might show a growth defect only at a pH and ethanolamine concentration for which the influx rate is limited by a low external Eth0 concentration. This was tested initially by scoring growth on solid medium.

Table 2 shows the effects of pH and ethanolamine concentration on the growth of wild-type and eutH mutant cells with ethanolamine as the carbon source. For each set of conditions, the calculated concentration of Eth0 is indicated. While the concentration of EutH varies that Eth0 may be needed at pH values sufficiently high to allow unaided diffusion. More detailed implications of these results are discussed later.

Table 2. Conditions under which a eutH mutant has a growth phenotype

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Growth* on ethanolamine as a carbon source at the indicated pH and concn of total ethanolamineb and Eth0c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH 5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 mM (2 μM)</td>
</tr>
<tr>
<td>TT22524</td>
<td>eutHΔ</td>
<td>0</td>
</tr>
<tr>
<td>TR10000</td>
<td>Wild-type LT2</td>
<td>4+</td>
</tr>
</tbody>
</table>

a Growth was scored qualitatively on an increasing scale from no growth (0) to strong growth (4+).

b Total concentration of ethanolamine in assay plates, regardless of charge.

c The concentration of Eth0 (in parentheses) was calculated for the indicated pH by using the Henderson-Hasselbach equation.
widely. Mutants lacking EutH were impaired for growth at Eth\(^\circ\) concentrations below about 25 \(\mu\)M. The concentration required by a eutH mutant is about 10-fold higher than that of wild-type cells (Table 3). The concentration required by EutH\(^+\) cells—about 3 \(\mu\)M ethanolamine—is close to the \(K_m\) (9 \(\mu\)M) of the first enzyme, ethanolamine ammonia lyase (24).

The growth ability of both wild-type and eutH mutant strains correlates with the external Eth\(^\circ\) concentration.

These growth responses were also seen in liquid growth tests (Fig. 4). At pH 7.5, wild-type and eutH mutant cells grow similarly at a range of ethanolamine concentrations (Fig. 4A and B). A reduced pH (Fig. 4C and D) impaired the ability of a eutH mutant to grow on lower ethanolamine concentrations but had no effect on the growth of wild-type cells (data not shown). It should be noted that at pH 7.5, the growth ability of parallel eutH mutant cultures was rather variable. We suggest that this reflects the frequent appearance of suppressor mutants that allow faster entry of ethanolamine.

A second test of EutH as a facilitator of ethanolamine uptake. It was previously suggested that ethanolamine catabolism releases sufficient acetaldehyde to inhibit the growth of cells that lack protective functions such as glutathione or the repair protein DNA polymerase I (14, 15). This suggested that conditions might be found under which added ethanolamine inhibits the growth of cells on an unrelated carbon source. If

![FIG. 4. Effect of pH on growth on ethanolamine as the sole carbon source. Solid and open symbols represent growth of wild-type LT2 and the eut\(\Delta\) mutant, respectively. Individual curves indicate growth on different initial concentrations of added ethanolamine. Symbols: squares, 20 mM; circles, 10 mM; triangles, 8 mM; diamonds, 6 mM. Parts A and B describe the growth of wild-type and eutH mutant cells at pH 7.5. Parts C and D describe the growth of a eutH mutant at pHs 7 and 6.5. Wild-type cells show the same growth response at pHs 6.5 and 7 (not shown) as at pH 7.5 (above).](http://jb.asm.org/)

**TABLE 3. Minimum ethanolamine for wild type**

<table>
<thead>
<tr>
<th>pH</th>
<th>Growth(^\circ) on ethanolamine as a carbon source at the indicated concn of total ethanolamine (mM)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>5.5</td>
<td>0 (0.026)</td>
</tr>
<tr>
<td>6.0</td>
<td>0 (0.10)</td>
</tr>
<tr>
<td>6.5</td>
<td>0 (0.26)</td>
</tr>
</tbody>
</table>

\(^a\) Growth on ethanolamine as the carbon source was scored qualitatively on an increasing scale from no growth (0) to strong growth (4+).

\(^b\) Total concentration of ethanolamine in plates regardless of charge. The concentrations (in micromolar units) of Eth\(^\circ\) at the indicated pHs were calculated by using the Henderson-Hasselbach equation and are presented in parentheses.
EutH contributes to ethanolamine influx, it should also contribute to this sensitivity under appropriate conditions.

Tests of this prediction were done with an in-frame eutE deletion mutant that lacks the second step of the ethanolamine pathway (Fig. 1) and should accumulate acetaldehyde produced from ethanolamine. This mutant grew as well as the wild type on succinate. Unlike the wild type, this mutant was inhibited for growth on succinate by added ethanolamine. The toxicity of ethanolamine required the first enzyme of the pathway.

That is, toxicity was eliminated by lack of the lyase cofactor B_{12} and by mutations that inactivate lyase (eutBC), suggesting strongly that acetaldehyde is responsible. We have also demonstrated that acetaldehyde is released by strains growing on ethanolamine (Penrod, unpublished). This allowed a second test of the suggested role of EutH in transport.

If EutH is involved in ethanolamine transport, it should contribute to the toxic effect of ethanolamine (described above) on a eutE mutant. That is, under conditions that require EutH for transport, a eutH mutation should reduce the toxic effect of ethanolamine. Conversely, if EutH acts at some later step in ethanolamine metabolism or if it facilitates efflux of toxic acetaldehyde, one might expect a eutH mutation to increase the toxicity of ethanolamine.

Rates of growth in liquid support the idea that EutH contributes to ethanolamine influx (Fig. 5). A eutH mutation did not impair the growth of a eutE mutant on succinate. However, as shown in Fig. 5, a eutH mutation substantially relieved the toxic effect on an eutE mutant of 10 mM ethanolamine at pH 7.0. As predicted if only Eth^{0} enters cells, the same eutH mutation had no effect at pH 7.5 and completely corrected ethanolamine sensitivity at pH 6.5 (data not shown).

**EutH protein is localized in the cell membrane.** The EutH sequence included 8 to 10 putative transmembrane segments (Fig. 2), suggesting that the protein might be inserted in the membrane, as expected for a transporter. The predicted location of EutH was tested with a fusion of yellow fluorescent protein (Yfp) to EutH. The yfp coding sequence was fused to the eutH gene within the eut operon as described in Materials and Methods. This chimeric gene produces a hybrid protein with the Yfp sequence linked to the C-terminal end of the EutH protein with a linker of 10 glycine residues. The EutH-Yfp fusion strain grew like the wild type in the assay described in Table 2, indicating that the fusion protein supplied normal EutH function and the construction caused no polar effects on downstream genes in the operon. Fluorescence microscopy revealed the localization of the EutH-Yfp protein near the cell periphery (Fig. 6). The specificity of this labeling is clear since the same Yfp sequence showed uniform labeling across the cell when fused to other proteins and labeled only points within the cell (thought to be carboxysomes) when fused to carboxysome shell proteins.

**DISCUSSION**

Cell swelling experiments showed a pH-dependent diffusion of ethanolamine into cells of *S. enterica*, indicating that the uncharged form Eth^{0} diffused freely across the membrane while the protonated form was excluded. This suggested that the putative ethanolamine transporter EutH might facilitate influx of the uncharged species and thus be needed only when the Eth^{0} concentration was reduced by a low pH and ethanolamine.

**Evidence that EutH is a diffusion facilitator for uncharged ethanolamine.** (i) In previous experiments, isotopes from labeled ethanolamine did not accumulate in cells without an intact degradative pathway (18). (ii) Wild-type cells use ethanolamine only when the Eth^{0} concentration is above 3 μM, a concentration near the *K_{m*} of ethanolamine ammonia lyase (9 μM). (iii) A eutH mutant failed to grow normally at Eth^{0} concentrations below 25 μM, suggesting that at lower concentrations the gradient provided insufficient flux for growth without facilitation. (iv) At physiological pH values, wild-type cells require an Eth^{0} concentration that approximates the *K_{m*} of the first enzyme.

Selective maintenance of the eutH gene in natural popula-
tions suggests that S. enterica frequently encounters ethanolamine at concentrations or under pH conditions that limit the external Eth\(^{0}\) level to between 3 and 25 \(\mu\)M—the range over which EutH enhances the ability to utilize ethanolamine.

The lack of an active transport system for ethanolamine may have several explanations. (i) The small amount of energy obtained from a two-carbon compound may make the cost of active transport prohibitive. Much of the derived energy is needed to generate larger carbon-containing molecules. Because ethanolamine is metabolized via the tricarboxylic acid cycle and glyoxalate shunt, formation of large carbon-containing molecules requires formation of phosphoenolpyruvate either from oxaloacetate (at a cost of one ATP) or from malate through pyruvate (at a cost of two ATPs). (ii) Much of the transported ethanolamine is lost as the volatile intermediate acetaldehyde (Penrod, unpublished), and some is likely to be lost as ethanol (formed to balance redox). (iii) Perhaps most importantly, actively transported ethanolamine would be subject to immediate loss because the uncharged form can cross the membrane by nonspecific diffusion.

**Interpretation of cell swelling assays.** The cell swelling assays in Fig. 3 have some curious features. While increased pH allowed more rapid swelling, the major effect is seen at pH values (pHs 9.5 to 10.5) that are above the pK for ethanolamine (pH 9.5), a range in which the external Eth\(^{0}\) concentration changes less than twofold; a much smaller effect is seen in the pH range of 7.5 to 9.5, over which the Eth\(^{0}\) concentration increases about 50-fold. Furthermore, even at the highest pH tested, the influx rate is slow compared to that seen for uncharged solutes such as glycero1 (5, 9) and is not affected by the presence of EutH. Generally these assays have been used to study the influx of nonionic solutes while here an ionizable compound is used. We suggest that EutH is a low-capacity channel and at these high concentrations, Eth\(^{0}\) enters at a slow rate, primarily through general unfacilitated routes. After entry, ethanolamine is reprotinated within the small cell volume, causing an increase in the internal pH that limits protonation of ethanolamine and reswelling. Full swelling requires reduction of the internal pH to that of the outside, which may be easier to achieve when the external pH is higher. During growth, the pH problems may be solved in part by metabolism of ethanolamine and attendant release of excess ammonia from the cell.

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**REFERENCES**