

Escherichia coli Glutamate- and Arginine-Dependent Acid Resistance Systems Increase Internal pH and Reverse Transmembrane Potential

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Due to the acidic nature of the stomach, enteric organisms must withstand extreme acid stress for colonization and pathogenesis. *Escherichia coli* contains several acid resistance systems that protect cells to pH 2. One acid resistance system, acid resistance system 2 (AR2), requires extracellular glutamate, while another (AR3) requires extracellular arginine. Little is known about how these systems protect cells from acid stress. AR2 and AR3 are thought to consume intracellular protons through amino acid decarboxylation. Antiporter mechanisms then exchange decarboxylation products for new amino acid substrates. This form of proton consumption could maintain an internal pH (pH_i) conducive to cell survival. The model was tested by estimating the pH_i and transmembrane potential ($\Delta\Psi$) of cells acid stressed at pH 2.5. During acid challenge, glutamate- and arginine-dependent systems elevated pH_i from 3.6 to 4.2 and 4.7, respectively. However, when pH_i was manipulated to 4.0 in the presence or absence of glutamate, only cultures challenged in the presence of glutamate survived, indicating that a physiological parameter aside from pH_i was also important. Measurements of $\Delta\Psi$ indicated that amino acid-dependent acid resistance systems help convert membrane potential from an inside negative to inside positive charge, an established acidophile strategy used to survive extreme acidic environments. Thus, reversing $\Delta\Psi$ may be a more important acid resistance strategy than maintaining a specific pH_i value.

Enteric organisms that colonize and cause disease in the human intestine must first endure a transient but extreme acid challenge in the stomach. The normal human stomach presents an antimicrobial acid environment averaging pH 2, with an emptying time of approximately 2 h (53). As a result, acid-sensitive pathogens like *Vibrio cholerae* must be ingested in massive numbers (10 to 100 million) to increase the possibility that some will survive and enter the intestine. Other microbes, such as *Escherichia coli* and *Shigella*, can colonize or cause disease even when small numbers of cells (10 to 100) are ingested. These resilient microbes are equipped with potent acid resistance systems able to withstand pH 2 challenge for at least 2 h (11, 31, 32, 52). In fact, *E. coli* possesses a level of acid resistance rivaling that of the gastric pathogen *Helicobacter pylori* (39, 45, 50, 59).

It has now been shown that *E. coli* uses four inducible acid resistance systems to survive extreme acid environments. Acid resistance system 1 (AR1), also referred to as the oxidative or glucose-repressed system, is acid induced in stationary phase. Its expression requires the alternative sigma factor RpoS and the cyclic AMP receptor protein CRP (11). However, the structural components of AR1 as well as the mechanism by which it protects are still unknown. The second AR system, AR2, requires extracellular glutamate to work at pH 2.0 and is induced upon entry into stationary phase or by log-phase growth in acidic minimal medium (10). Known components of

glutamate-dependent acid resistance include two isoforms of glutamate decarboxylase (GadA and GadB) and a putative glutamate: γ -aminobutyric acid (GABA) antiporter called GadC (11, 12, 19, 33, 46). The third system, AR3, is similar to AR2 except that AR3 only protects cells if extracellular arginine is present. AR3 is induced by low pH under anaerobic conditions and has only been demonstrated following growth in complex media. This arginine-dependent system is composed of the acid-inducible arginine decarboxylase AdiA and the AdiC antiporter, which exchanges extracellular arginine for the intracellular end product of decarboxylation, agmatine (11, 15, 22, 31). The last AR system was recently described as lysine dependent and probably involves the inducible lysine decarboxylase (22).

Although it is clear that these systems protect *E. coli* during transient exposure to pH 2, how they actually function has been subject to speculation. It is believed that AR2 and AR3 protect the cell from acid stress by consuming intracellular protons during each decarboxylation reaction (11, 14). The siphoning off of intracellular protons was proposed to enhance pH homeostasis and allow the cell to maintain an internal pH compatible with viability. This model suggests that a specific internal pH may be crucial for survival during exposure to extreme acid stress. If the cell's internal pH fell below that point, it would succumb. The data obtained in the present study indicate that maintenance of a specific internal pH may not be paramount to cell survival. Survival may depend on *E. coli* taking an approach used by acidophiles, which is to reverse the electrical membrane potential ($\Delta\Psi$) in the presence of extreme low pH. Converting membrane potential from nega-

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live inside to positive inside can repel protons and mitigate the excess proton motive force (PMF) that can form.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study included EK227, wild-type K-12; EK592, wild-type MG1655; EK590, $\Delta clcB \Delta clcA$ (derived from MG1655 [24]); EF333, $gadC::Tn10$ (3); E1522, $gadA::pRR10$ (AP) $gadB::Tn10$ (3); and EF996, $\Delta uncH-C::Tn10$ (derived from EK227). Media included Luria-Bertani broth (LB) and brain heart infusion (BHI) medium containing 0.4% glucose (LBG and BHIG). LB broth, where indicated, was buffered with either 100 mM morpholinopropanesulfonic acid (MOPS; pH 8.0) or morpholineethanesulfonic acid (MES; pH 5.0). For internal pH measurements, these media also contained 25 mM sucrose to block nonspecific binding of radiolabeled sucrose (see below). Acid challenge medium was minimal EG (58) prepared at various pH values (adjusted with HCl). For the reasons noted above, EG also contained 25 mM sucrose when used for internal pH measurements. To test whether Na^+ - or K^+ -deficient medium was important, Milli-Q Ultrapure water was prepared at pH 2.5 either with or without 1 mM glutamic acid \cdot HCl. In addition, a Na^+ - and K^+ -deficient medium (M63 K/Na-deficient) containing 15 mM $(NH_4)_2SO_4$, 18 μ M $FeSO_4 \cdot 7H_2O$, 1 mM $MgSO_4$, and 0.2% glucose was used. All chemicals used were ultrapure (Sigma or Fluka) or Suprapur (EM). Na^+ and K^+ measurements were made using sodium and potassium ion-specific combination electrodes (Thermo-Orion). Cultures were grown at 37°C with shaking at 220 rpm. Exponential-phase cultures were grown to an optical density at 600 nm of 0.4 (2×10^8 CFU/ml), while stationary-phase cultures were grown overnight (18 h).

Acid resistance assays. Acid resistance assays were performed as described previously (11). Briefly, cells were grown overnight in LB MOPS and LB MES for AR1, LBG for AR2, or BHIG for AR3. LBG was used when studying the glutamate-dependent system, but since arginine decarboxylase is not induced well in LBG, BHIG was used to induce this system. Stationary-phase cultures were diluted 1:1,000 into prewarmed pH 2.5 EG medium without amino acid supplementation (for AR1), with 1.6 mM glutamate (for AR2), or with 1.0 mM arginine (AR3). At various time points, 10- μ l aliquots were removed and serially diluted, and 10 μ l of each dilution was plated on LB plates. CFU were determined, and percent survival was calculated relative to time zero.

Internal pH measurements. Internal pH was estimated by measuring the distribution of a weak acid (radiolabeled salicylic acid) across the membrane (4, 8, 23). Salicylate has been used by us and others as a reliable indicator of internal pH (13, 24, 26, 34, 47). Control cultures were grown to log phase (2×10^8 CFU per ml) or stationary phase (10^8 CFU per ml) in LBG containing 25 mM sucrose (final pH at time of assay, 6.9). Sucrose was added to prevent nonspecific binding of radiolabeled sucrose, used later for water space measurements. Cultures to test decarboxylase-dependent effects on internal pH were grown overnight to stationary phase in LBG containing 25 mM sucrose for AR2 measurements or in BHIG containing 25 mM sucrose for AR3 measurements. Cultures were then harvested by centrifugation and resuspended in pH 7 EG medium containing no additions or in pH 2.5 EG medium with and without 40 mM glutamate or arginine. Final cell density after resuspension was 6×10^9 CFU/ml. Two reactions were required for each assay. A total of 2,000 to 3,000 dpm of 3H_2O / μ l (0.1 μ Ci/ μ l) was added to each reaction mixture. Next, 2,000 to 3,000 dpm of [^{14}C]salicylate (56.5 mCi/mmol) was added to one tube, and the same amount of [^{14}C]sucrose (462 mCi/mmol) was added to the other at time zero. At specific times (30 or 60 min), 5 μ l was taken from each tube for a direct isotope count, and 100- μ l aliquots were centrifuged through equal amounts of dibutylphthalate (50 μ l) and silicone oil (50 μ l) to separate the supernatant from the cell pellet. The amount of [^{14}C]sucrose, which does not penetrate the cytoplasmic membrane, was used to determine the extracellular and periplasmic water space remaining after centrifugation. Disintegrations per minute for [^{14}C]salicylate and 3H_2O were then used to determine the internal pH value by the following equation: $pH_i = \log\left(\frac{[A_{out}]/[A_{in}]}{(10^{pK_a} + 10^{pH_{out}}) - 10^{pK_a}}\right)$, where $[A]$ is a measure of salicylic acid and the pK_a is 3.0.

Ψ measurements. $\Delta\Psi$ was measured using radiolabeled lipophilic anions or cations (4, 23). Cells were grown as for internal pH measurements. Log-phase and stationary-phase cultures were harvested by centrifugation and resuspended in pH 2.5 EG medium containing 40 mM glutamate or 40 mM arginine. Final cell densities were approximately 6×10^9 CFU per ml. Each measurement involved two assays, one for charge distribution and one to determine water space. The general methodology was similar to that used to estimate internal pH. For charge distribution, 1,200 dpm of [^{14}C]tetraphenylphosphonium bromide (TPP $^+$; 5 mCi/mmol) or potassium [^{14}C]thiocyanate ($S^{14}CN^-$; 54 mCi/mmol)/ μ l was added to one tube along with 2,000 to 3,000 dpm of 3H_2O . Extracellular and intracellular

water spaces were determined as above. Extracellular water space was used to correct for the carryover of extracellular radiolabeled lipophilic ion not removed during centrifugation. At 30 min, 5 μ l was taken for total direct counts and 100 μ l was centrifuged through dibutylphthalate-silicone oil. The accumulations of [^{14}C]TPP $^+$ (or $S^{14}CN^-$) and 3H_2O in cell pellets were used to determine $\Delta\Psi$ (23) by the following equation: $\Delta\Psi = RT/zF \cdot \ln\left(\frac{[A_{out}]/[A_{in}]}{[A_{out}]/[A_{in}]}\right)$, where R is the gas constant (8.28 J/K \cdot mol), T is temperature (310.15 K), z is the charge of the molecule (+ or -), and F is the Faraday constant (96,485 J/V \cdot mol).

Similar assays were done with butanol-treated cells (20% butanol) to determine nonspecific binding, which was subtracted as background from the experimental results. Background counts were no more than 10% of experimental counts.

Whole-cell decarboxylation and antiport measurements. Transport and conversion of [3H]arginine and [3H]glutamate to [3H]agmatine or [3H] γ -aminobutyric acid, respectively, and the subsequent end product efflux were measured using intact and Triton X-100-permeabilized cells. Wild-type and $gadA/B$ and $gadC$ mutant cells were grown for 22 h in 3 ml of BHIG (for arginine decarboxylation) or LBG (for glutamate decarboxylation). Cells were harvested by centrifugation, washed twice with EG medium (pH 7.0), and resuspended to 10^8 cells/ml in 3.0 ml of prewarmed EG medium adjusted to pH 2.5 with HCl or to other pH values as indicated. The medium contained 1.0 mM arginine, including 4 μ Ci of [3H]arginine (61 Ci/mmol) per ml or a final concentration of 0.4 mM glutamate including 22,000 dpm of [3H]glutamate/ μ l. At timed intervals, 500- μ l aliquots of cell-free supernatants were obtained by filtration (0.45- μ m-pore-size filters) and adjusted to pH 7.5 with 5 N NaOH, and 30- μ l samples were subjected to paper chromatographic separation as described previously (29). Marked bands were cut and counted for radioactivity. Percent conversion was calculated from total radioactivity on each strip.

Western blot analysis and cellular location of GadC. Cells were grown overnight in 50 ml of EG pH 7.7 and EG pH 5.5. The cells were harvested by centrifugation at $4,500 \times g$ for 10 min (4°C), resuspended in 5 ml of cold 10 mM HEPES buffer (pH 7.4), and passed twice through a French pressure cell (Aminco) at 16,000 lb/in 2 . Crude extracts were cleared of cell debris by centrifugation at $4,500 \times g$ for 10 min (two times). The resulting cleared supernatant was ultracentrifuged at $90,000 \times g$ (4°C) to separate membrane and soluble fractions. Membrane pellets were washed with 2.0 ml of 10 mM HEPES buffer (pH 7.4) to remove residual soluble proteins and resuspended in 300 μ l of the same buffer. Soluble fractions were also centrifuged at $90,000 \times g$ to remove residual membrane. Protein concentrations were measured using the Bio-Rad protein assay reagent.

Western blot analysis of these fractions was carried out using rabbit anti-GadC antibodies raised against a GadC-specific peptide (N^1 -CRARSPHYIV MNDKKH) by Genemed Synthesis, Inc. Membrane and soluble fractions (3 μ g of protein) were separated on 10% polyacrylamide-sodium dodecyl sulfate gels (30). Proteins were transferred to Immobilon-P (polyvinylidene difluoride) membranes with a Semiphore transfer cell (Hofer Scientific) at 100 mA for 2 h. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (10 mM Tris [pH 8], 150 mM NaCl) containing 0.05% Tween 20 and incubated with rabbit primary (1:8,000) and mouse anti-rabbit secondary (1:8,000) antibodies for 1 h at room temperature. The blot was developed with ECL detection reagents (Amersham Pharmacia Biotech).

RESULTS

The role of Cl^- transporters and the F0/F1 proton-translocating ATPase in acid resistance. The goal of this study was to further define how the amino acid-dependent AR systems protect against acid stress. We initially wanted to determine how similar these systems were to the amino acid-independent system AR1, whose mechanism is also a mystery. An elegant study by Iyer et al. previously demonstrated that Cl^- transporters encoded by the *clc* genes of *E. coli* were important for AR2 and AR3 function (1, 21). However, their potential role in system 1 was not explored. Consequently, we examined whether the *Clc* transporters also contributed to AR1.

Wild-type and $\Delta clcA/B$ cells were grown to stationary phase in LB MES pH 5.5, an inducing condition for AR1, and then tested for survival in pH 2.5 minimal medium without amino acid supplementation (Fig. 1A). Both strains exhibited normal

