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YjdE (AdiC) Is the Arginine:Agmatine Antiporter Essential for Arginine-Dependent Acid Resistance in *Escherichia coli*

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To survive in extremely acidic conditions, *Escherichia coli* has evolved three adaptive acid resistance strategies thought to maintain internal pH. While the mechanism behind acid resistance system 1 remains enigmatic, systems 2 and 3 are known to require external glutamate (system 2) and arginine (system 3) to function. These latter systems employ specific amino acid decarboxylases and putative antiporters that exchange the extracellular amino acid substrate for the intracellular by-product of decarboxylation. Although GadC is the predicted antiporter for system 2, the antiporter specific for arginine/agmatine exchange has not been identified. A computer-based homology search revealed that the *yjdE* (now called *adiC*) gene product shared an overall amino acid identity of 22% with GadC. A series of *adiC* mutants isolated by random mutagenesis and by targeted deletion were shown to be defective in arginine-dependent acid resistance. This defect was restored upon introduction of an *adiC*$^+$-containing plasmid. An *adiC* mutant proved incapable of exchanging extracellular arginine for intracellular agmatine but maintained wild-type levels of arginine decarboxylase protein and activity. Western blot analysis indicated AdiC is an integral membrane protein. These data indicate that the arginine-to-agmatine conversion defect of *adiC* mutants was at the level of transport. The *adi* gene region was shown to be organized into two transcriptional units, *adiAY* and *adiC*, which are coordinately regulated but independently transcribed. The data also illustrate that the AdiA decarboxylase: AdiC antiporter system is designed to function only at acid levels sufficient to harm the cell.

Orally ingested enteric bacteria seeking to breach the gastric barrier and gain entrance to the intestine come under lethal attack from stomach acidity. Some species are poorly equipped to handle this stress and require massive assaults, involving billions of cells, in the hope that a few survivors gain their objective (e.g., *Vibrio cholerae*). Other microbes are armed with potent acid resistance mechanisms that enable small numbers of bacteria to slip through the stomach unscathed. Pathogenic and nonpathogenic (natural) strains of *Escherichia coli* possess three distinct acid resistance systems whose redundancy allows for an oral infectious dose of less than 100 ingested organisms. The three acid resistance systems, designated AR 1, AR 2, and AR 3, have unique induction signatures and employ different mechanisms to provide low pH protection. All systems work best in stationary-phase cells.

AR 1 is produced by Luria-Bertani (LB)-grown, stationary-phase cells and protects *E. coli* at pH 2.5 in simple, defined minimal medium (3, 4). It seems to be expressed regardless of growth pH, but the activity is blocked by a diffusible inhibitor produced during growth under alkaline pH (pH 8). Expression of AR 1 is glucose repressed, and the protective mechanism remains undefined.

AR 2 has been the most intensely studied of the three systems. It requires glutamic acid to protect cells during pH 2.5 acid challenges. Two isoforms of a pyridoxyl phosphate-containing enzyme, glutamate decarboxylase, convert glutamic acid to γ aminobutyric acid (GABA) in a process that consumes an intracellular proton. Based on sequence homology to other amino acid antiporters, GadC, a predicted inner membrane protein, is thought to recruit glutamate from the medium in exchange for expelling GABA. The coupling of antiport to decarboxylation is predicted to drain protons from the cytoplasm, helping to maintain internal pH and/or proton motive force under extreme acid stress. Regulation of the *gad* system is very complex, involving two AraC-like proteins, two repressors (CRP and H-NS), and two sigma factors. The *gad* genes are induced at pH 5 in log-phase cells or in stationary-phase cells regardless of pH (3).

The third acid resistance system requires arginine to protect cells at pH 2.5. It appears to function much like system 2. Of critical importance is the *adiA* gene encoding the inducible form of arginine decarboxylase (ADC) (4, 10). This enzyme decarboxylates arginine to agamatine in a mechanism similar to that of glutamate decarboxylase. The ADC gene is highly induced under anaerobic conditions in rich medium at low pH (1, 16). Mutations in *adiA* selectively eliminate arginine-dependent acid resistance without affecting the other two systems (4). However, the requisite arginine:agmatine antiporter has not been identified. In this report, an open reading frame, *adiC (yjdE)*, located downstream of *adiA*, was identified as this antiporter.

MATERIALS AND METHODS

Strains, plasmids, oligonucleotides, and growth medium. The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. The minimal medium used was E medium containing 0.4% glucose (19). E medium is composed of 73 mM K_2HPO_4, 17 mM $NaNH_4HPO_4$, 0.8 mM $MgSO_4$, and 10 mM citrate. The complex medium used was brain heart infusion (BHI) broth containing 0.4% glucose (BHIG) and LB composed of (per liter) 10 g of Bacto-

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TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Name	Genotype or characteristics	Reference or source
Strains		
EK227	K-12 wild type λ^- F^-	A. C. Matin
EF336	K12 *adiA*::MudJ	EK198 × EK227
EK420 (BW25113)	pKD46/Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrn* B-4) *lacIp4000*	B. Wanner
EK445 (C600)	*thr-1 leuB6 thi-1 lacY-1 glnV44 rfbD1 fhuA21 supE*	M.-P. Castanie-Cornet
EF865	EK227 Δ(*gadXW*::Km) Δ*crp*::Cm	12
EF1011	EK420 Δ*adiC*1::Km	This study
EF1021	EK227 Δ*adiC*1::Km	EK227 × EF1011
EF1023	Δ(*yhiXW*::Km) Δ*crp*::Cm *adiC* 2::Tn*10*dTc	EF865 × λ1098
EF1024	K-12 Δ(*yhiXW*::Km) Δ*crp*::Cm *adiC* 3::Tn*10*dTc	EF865 × λ1098
EF1025	K-12 Δ(*yhiXW*::Km) Δ*crp*::Cm *adiC* 4::Tn*10*dTc	EF865 × λ1098
EF1026	K-12 Δ(*yhiXW*::Km) Δ*crp*::Cm *adiC* 5::Tn*10*dTc	EF865 × λ1098
EF1028	K-12 Δ(*yhiXW*::Km) Δ*crp*::Cm *adiC* 6::Tn*10*dTc	EF865 × λ1098
EF1027	K-12 Δ(*yhiXW*::Km) Δ*crp*::Cm *adiC* 7::Tn*10*dTc	EF865 × λ1098
EF1029	K-12 Δ(*yhiXW*::Km) Δ*crp*::Cm *adiC* 8::Tn*10*dTc	EF865 × λ1098
EF1030	K-12 Δ(*yhiXW*::Km) Δ*crp*::Cm *adiC* 9::Tn*10*dTc	EF865 × λ1098
EF1045	INVαF′/pSGF520 Ap^r	This study
EF1050	INVαF′/pSGF523, Ap^r	This study
EF1051	K-12 Δ*adiC*::Km/pBAD24 Ap^r	EF1021 × pBAD24
EF1052	K-12 Δ*adiC*::Km/pSGF523 Ap^r	EF1021 × pSGF523
EF1053	K-12 Δ*adiC*::Km/pSGF526 Ap^r	EF1021 × pSGF526
EF1054	INVαF′/pSGF526 Ap^r	This study
Plasmids		
pCR2.1	TA cloning vector	QIAGEN
pSGF520	1,734-bp fragment containing $adiC^+$ cloned into pCR2.1; Ap^r	This study
pBAD24	4.5-kb cloning vector; Ap^r	Guzman
pSGF523	1,842-bp *Xba*I/*Hin*dIII fragment from pSGF520 cloned into pBAD24; Ap^r	This study
pSGF526	1,818-bp *Kpn*I/*Xho*I fragment from pSGF520 cloned into pBAD24; Ap^r	This study
Oligonucleotides		
oligo-51	5′-GAC AAG ATG TGG ATC CAC CTT AAC-3′; inverted repeat region of Tn*10*(dTc)	
oligo-96	5′-CCG GGT AAG CAA TGA TGA AAG TA-3′; begins at the *adiA* start codon	
oligo-97	5′-TTA CGC TTT CAC GCA CAT AAC G-3′; begins at the *adiA* stop codon	
oligo-404(k2)	5′-CGG TGC CCT GAA TGA ACT GC-3′; common test primer used to verify pKD13 Km insertion	B. Wanner
oligo-405(Kt)	5′-CGG CCA CAG TCG ATG AAT CC-3′; common test primer used to verify pKD13 Km insertion	B. Wanner
oligo-467	5′-TGT CTT CGG ATG CTG ATG CTC ACA AAG TGG GCT TAATCC GTG TAG GCT GGA GCT GCT TCG-3′; used to create the Δ*adiC*::Km mutation	
oligo-468	5′-TTA ATC TTT GCT TAT TGG TGC ATC TAA GGG ATA CGGGTT TAT TCC GGG GAT CCG TCG ACC-3′; used to create the Δ*adiC*::Km mutation	
oligo-481	5′-TTC GGC AAA TAT GTC GGC ATG-3′; used for checking the insertion of Tn*10*(dTc) in *adiA*	
oligo-483	5′-GAC GGT ATT TAC CAC GTT ATG-3′; used to verify the *adiC* deletion	
oligo-484	5′-ACA TGT ACT CCT GAG TGC GAA-3′; used to screen Tn*10*(dTc) insertions in *adiA*	
oligo-488	5′-CGA ACA AAG TGC GCA TAT GCT-3′; used to screen Tn*10*(dTc) insertions in *adiC*	
oligo-505	5′-GCA TGC GTT ATG CCG TAA ATG-3′; used to amplify *adiC*	
oligo-506	5′-CCA GTA GAG GAC GTT GGT TTG-3′; used to screen Tn*10*(dTc) insertions in *adiY*	
oligo-578	5′-TTT TCA CCA CAC CTG CGG CAA-3′; used to amplify *adiC* internal fragment	
oligo-579	5′-ATG TCT TCG GAT GCT GAT GCT-3′; used to amplify *adiC*	

Tryptone, 5 g of Bacto-Yeast Extract, and 5 g of NaCl. LB broth, where indicated, was buffered to 100 mM with either MOPS (morpholinepropanesulfonic acid, pH 8.0) or MES (morpholineethanesulfonic acid, pH 5.5). Liquid ADC medium included, per liter, 5 g of Bacto peptone, 5 g of Bacto beef extract, 0.5 g of D-glucose, and 10 g of L-arginine. ADC medium was adjusted to pH 5.5 or 8 with HCl or NaOH. SOB and SOC media were described elsewhere (7). Restriction enzymes were purchased from Promega Biotech.

Cultures were typically grown under semiaerobic conditions (3 ml of medium in 13- by 100-mm test tubes, shaking at 240 rpm and 37°C). Anaerobic conditions were imposed with a filled screw cap culture tube. The following antibiotics were used as needed: ampicillin (Ap) at 60 μg/ml, kanamycin (Km) at 50 μg/ml, tetracycline (Tc) at 30 μg/ml, and chloramphenicol (Cm) at 40 μg/ml.

Construction of a λ1098 random mutagenesis library. Bacteriophage λ1098 containing mini-Tn*10*::Tet was propagated on EK445 and used for transposition as described previously (13, 17). Single colonies of the target strain (EF865) arising on tetracycline-containing LB plates (42°C) were inoculated into 96-well plates containing LB–15% glycerol and incubated for 6 h at 30°C before entering frozen storage (−80°C).

Screening of the λ1098 random mutagenesis library. Separately, 7,800 random Tn*10*dTc clones were cultured in microtiter plate wells containing BHI with 0.4% glucose (suitable for inducing AR 3). Cultures were incubated anaerobically with BBL GasPaks for 22 h (37°C). The microtiter plate cultures were then replicated into liquid E glucose (EG) medium at pH 2.5. After 0, 8, and 10 h of acid challenge, surviving cells were rescued onto tetracycline-containing LB agar and incubated overnight. Acid-sensitive mutants were selected as clones that failed to survive 8 h of acid challenge.

Construction of a Δ*adiC* (Δ*yjdE*) mutant. The one-step method of gene inactivation was used to create a targeted deletion of *adiC* (*yjdE*) (5). A 1.4-kb PCR product needed to create the deletion was made from oligo-467 and oligo-468, which include 40 nucleotides at their 5′ ends that are homologous to the ends of *adiC* and 20-nucleotide priming sequences for the Km^r gene of pKD13 at the 3′ ends. PCR products were gel purified, digested with *Dpn*I, repurified, and electroporated into EK420 containing *red* recombinase. Putative Km^r Δ*adiC* mutants were maintained on medium without an antibiotic to enable loss of the *red* helper plasmid. Verification of the mutation was made by PCR using locus-specific primers (oligo-505 and oligo-488) and common test primers (oligo-404 and

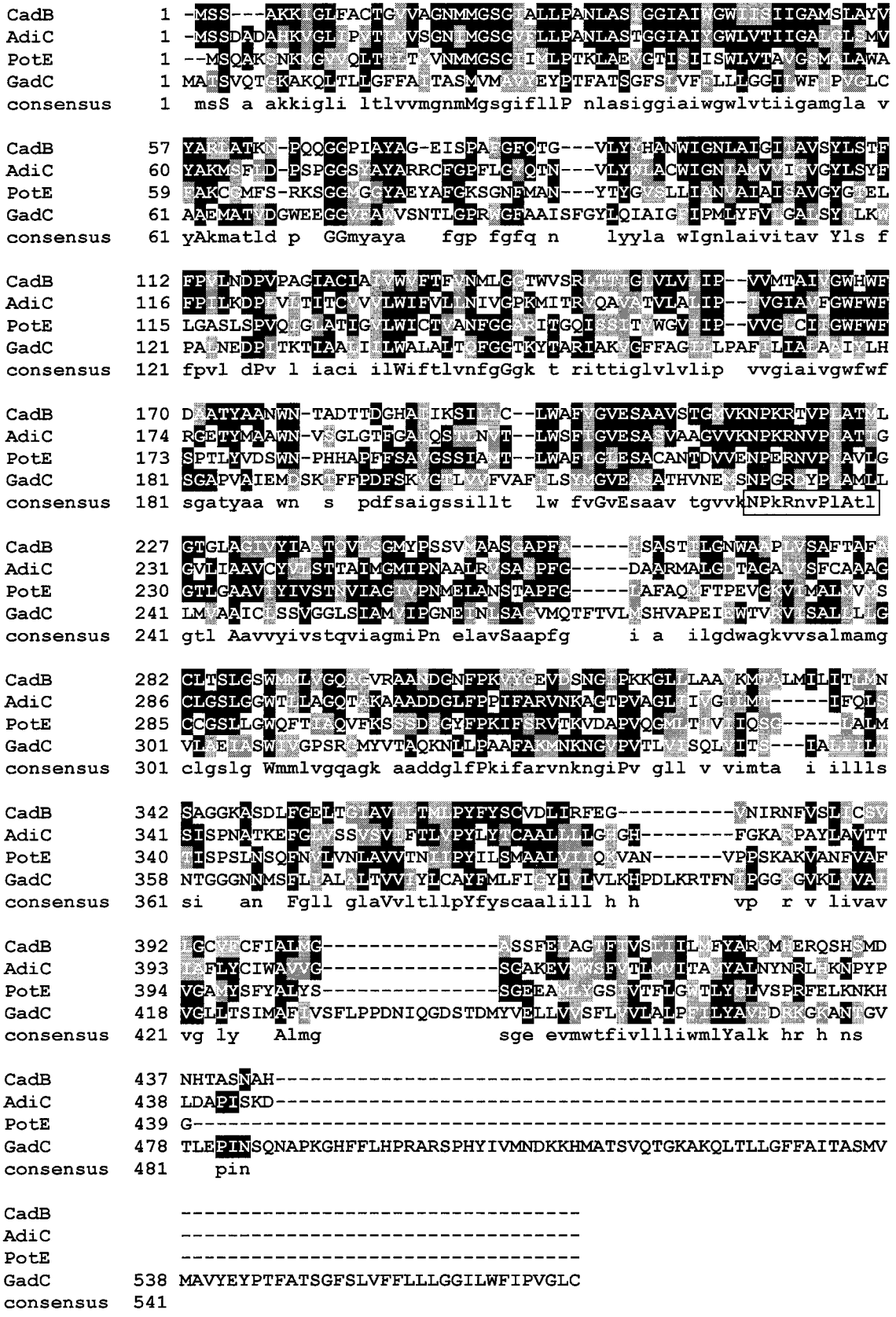

FIG. 1. Homology-based prediction of a putative arginine:agmatine antiporter. CLUSTAL W amino acid sequence alignments of AdiC (YjdE), the predicted arginine:agmatine antiporter; CadB, the lysine:cadeverine antiporter of the lysine decarboxylase system; PotE, the ornithine:putrescine antiporter of the ornithine decarboxylase system; and GadC, the putative glutamate:GABA antiporter of the glutamate decarboxylase system. Identical amino acids are shown in black boxes, while similar amino acids are shown in gray boxes. The consensus line shows identical (uppercase) and similar (lowercase) amino acids.

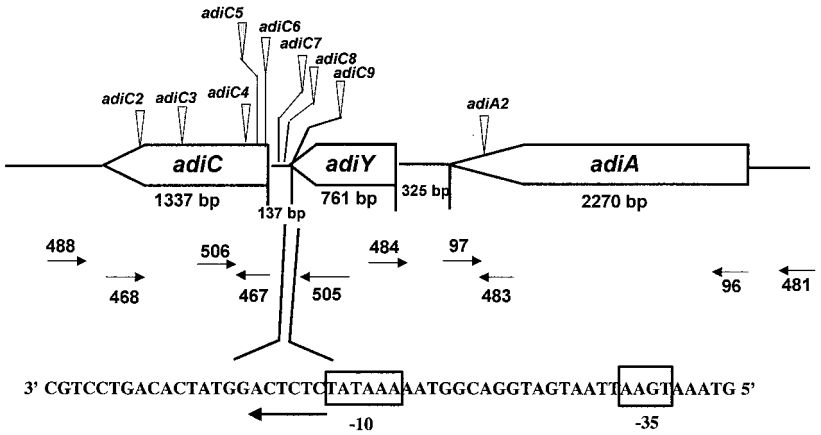

FIG. 2. Genetic organization of the *E. coli adi* cluster and the locations of acid sensitive Tn*10*dTc insertions. Block arrows representing genes point in the direction of transcription. The small arrows indicate relative binding locations of the oligonucleotide primers used in this study. Vertical arrowheads represent approximate positions of acid-sensitive Tn*10*dTc insertions as determined via PCR analysis. Each designation represents a class of Tn*10* insertions. The inset sequence at the bottom shows a predicted *adiC* promoter as determined by using the Neural Network Promoter Prediction site (http://www.fruitfly.org/seq_tools/nnppHelp.html). Putative −10 and −35 regions are marked.

oligo-405). The mutation was transduced into EK227 by P1 transduction, creating EF1021.

Acid resistance assays. To test for AR 1, cells were prepared by overnight growth in LB-MES (pH 5.5) and LB-MOPS (pH 8) for 22 h. LB containing 0.4% glucose was used to prepare cells to test AR 2, while cells grown in BHIG were used to test AR 3. The above three stationary-phase cultures were diluted 1:1,000 into prewarmed EG medium (pH 2.5) to test acid resistance (final cell concentration, 2×10^6/ml). Dilutions were made in unsupplemented EG medium (pH 2.5) for AR 1, EG medium (pH 2.0) supplemented with 0.7 mM glutamate for AR 2, and EG medium (pH 2.5) containing 1.5 mM arginine for system 3. Viable counts were determined at 0, 1, 2, and 4 h post-acid challenge.

Cloning of *adiC*. The *adiC* gene was amplified with *pfx* polymerase (Invitrogen) with oligo-505 and oligo-488. The reactions were run as described above, except the extension temperature used was 68°C. The 1,734-bp fragment was purified and cloned into pCR2.1 (TA cloning kit; QIAGEN) resulting in pSGF520. The 1,842-bp *Xba*I/*Hin*dIII and 1,818-bp *Kpn*I/*Xho*I fragments isolated from pSGF520 were cloned into pBAD24 (6), resulting in pSGF523, where *adiC* is oriented for expression from the *araBAD* promoter and where pSGF526 is oriented with the opposite orientation. These two plasmids, as well as the vector pBAD24, were then transformed into EF1021.

Western blot assay. Antibodies were raised in rabbits to peptide QYPDTYA NMGIHDLC for AdiA and peptide CLHKNPYPLDAPISKD for AdiC (YjdE) by Genemed Synthesis, Inc. Bacterial cultures for Western blot analysis were grown overnight in 3 ml of BHIG at 37°C with shaking. The 3-ml cell samples were harvested by centrifugation at 10,000 × *g* for 5 min, resuspended in 100 μl of 0.01% sodium dodecyl sulfate (SDS) sample buffer (9), and stored at −20°C. Protein concentration was measured by using Bio-Rad Protein Assay reagent. To examine AdiA, samples containing 5 μg of protein were boiled at 100°C for 5 min and loaded on 10% polyacrylamide-SDS minigels according to the method of Laemmli (9). Samples to examine AdiC, on the other hand, were not boiled because AdiC monomers aggregated in boiled preparations. Membranes were prepared through the ultracentrifugation (100,000 × *g*) of lysates cleared of debris by low-speed centrifugation. Proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) were transferred to Immobilon-P (polyvinylidene difluoride [PVDF]) membranes with a Semiphore transfer cell (Hoefer Scientific) at 100 mA for 2 h. The membrane was 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 (1:2,000) and mouse anti-rabbit secondary (1:3,000) antibody for 1 h at room temperature. The blot was developed with ECL detection reagents (Amersham Pharmacia Biotech).

Northern blot analysis. Cells were grown under anaerobic conditions (filled screw-cap tubes) to log phase (optical density at 600 nm, 0.4; 2×10^8 cells per milliliter) in ADC medium adjusted to pH 5.5 or 8.0. Total RNA was extracted by using the RNeasy kit (Qiagen). The RNA concentration was determined by measuring optical densities at 260 and 280 nm. Five micrograms of total RNA denatured at 65°C for 10 min was subjected to electrophoresis through a 1.0% denaturing formaldehyde-agarose gel, as described previously (14). The RNA was transferred onto a nylon membrane (Amersham-Pharmacia) and baked at 80°C for 2 h. The membranes were probed with a 0.656-kb *adiC* probe generated by PCR with oligonucleotides oligo-578 and oligo-579 or a 1.062-kb *adiA* probe made with oligonucleotides 103 and 104. Probes were labeled with [α-^{32}P]dCTP (Amersham) using the random-primed DNA kit (Ambion). The hybridizations were performed as described in the product literature.

Transport assays using whole cells. Transport of [^{3}H]arginine and conversion to ^{3}H-agmatine was assayed at 37°C. Wild-type and *adiA* and *adiC* mutant cells were grown in 3 ml of BHIG for 22 h, 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 a final arginine concentration of 1.0 mM, including 4 μCi of [^{3}H]arginine (61 Ci/mmol) per milliliter. At timed intervals, 500-μl aliquots were filtered through 0.45-μm-pore-size filters to collect cell-free supernatants. The supernatants were adjusted to pH 7.5, and 30-μl samples were used for paper chromatography. Chromatographic separation of amino acids and polyamines was conducted as described previously (8). Briefly, supernatant samples were spiked with unlabeled standards (L-arginine, agmatine) and spotted on Whatman No.1 chromatography paper. The strips were developed for 17 h in a descending manner with a solvent containing acetone (35 ml), butanol (35 ml), acetic acid (7 ml), and water (23 ml). Once developed, the paper strips were dried and sprayed with 0.3% ninhydrin to visualize the arginine and agmatine spots. The marked bands were cut and counted for radioactivity.

RESULTS

Bioinformatic identification of a potential arginine:agmatine antiporter. Prior this report, there were three known or suspected amino acid:polyamine antiporters (CadB, PotE, and GadC) in *E. coli* that shared a considerable amount of amino acid sequence similarity, including 29 identical amino acids. A BLAST search for other potential amino acid antiporters revealed that the deduced product of *yjdE* shares a 22% overall identity with the putative glutamate:GABA antiporter (GadC); 35% identity with the CadB lysine:cadaverine antiporter; and 29% identity with the ornithine:putrescine antiporter, PotE (Fig. 1). The *yjdE* gene is located directly downstream from *adiY* and *adiA*, as shown in Fig. 2. YjdE, which we have renamed AdiC, is a predicted 466-amino-acid protein possessing 12 putative transmembrane domains. These characteristics made AdiC (YjdE) a promising candidate for the sought-after arginine:agmatine antiporter.

Screening for mutants defective in arginine-dependent acid resistance. As described in Materials and Methods, a microtiter plate assay for arginine-dependent acid resistance was developed with EK227 (wild-type) and EF336 (*adiA*::MudJ). A total of 7,800 Tn*10*dTc insertion mutants were screened, and 72 potential acid-sensitive mutants were identified. Thirty of these acid-sensitive mutants were confirmed by the standard test tube acid resistance assay. The thirty confirmed acid-sensitive mutants were further analyzed by PCR to localize the insertions. The anchor oligonucleotide, oligo-51, which binds to the inverted repeat ends of Tn*10*, was used in combination with other oligonucleotides specific to various genes in the *adiAY adiC* (*yjdE*) region. Oligo-481 and oligo-484 were used to check for insertions in *adiA*, oligo-483 and oligo-506 detected insertions in *adiY*, and oligo-488 and oligo-505 identified *adiC* insertions (Fig. 2). Twenty-six of the 30 acid-sensitive mutants mapped to the *adi* gene cluster. Figure 2 illustrates the clustering of these insertions at eight locations in or immediately upstream of *adiC*. One insertion occurred within *adiA*, but there were no acid-sensitive insertions into *adiY*. The identities of the remaining mutants and their roles in acid resistance will be described elsewhere.

Computer analysis of the region between *adiY* and *adiC* revealed a potential promoter site approximately 270 bp from the AdiC start codon. The *adiC9*::Tn*10* insertion farthest upstream from the AdiC start codon occurred about 100 bp downstream of this predicted promoter, based on PCR analysis (Fig. 2).

Effect of *adiC* on arginine-dependent acid resistance. A complete deletion of *adi*C was constructed by using the *red* recombinase one-step inactivation protocol (Materials and Methods). This deletion mutant was tested for effects on all three acid resistance systems. The data in Fig. 3 clearly indicate that the Δ*adiC* strain was proficient in AR 1 and 2 (Fig. 3A and B) but was missing arginine-dependent AR 3 (Fig. 3C).

To rule out possible polar effects of the deletion scar left by the construction, we transformed the plasmids pBAD24, pSGF523 (*adiC*$^+$), and pSGF526 (*adiC*$^+$) into the Δ*adiC* strain and repeated the acid resistance assay. Plasmids pSGF523 and pSGF526 contain *adiC*$^+$ in opposite orientations relative to the arabinose promoter. Transformed cells were grown in BHIG with and without 1 mM arabinose for 22 h and challenged at pH 2.5 with and without arginine. Only the arginine results are shown. Figure 3D reveals that survival of the *adiC* mutant containing pSGF523 and pSGF526 were identical to that of wild-type EK227, while the mutant strain carrying vector alone did not survive the acid stress. All strains succumbed to pH 2.5 in the absence of arginine (data not shown). Thus, the arginine-dependent acid resistance defect of the *adiC* mutant can be attributed to the loss of *adiC* and not to polar effects on downstream genes. Furthermore, the data suggest the *adiC*$^+$ cloned region contains a dedicated *adiC* promoter, since insertions in either orientation successfully complemented the mutation. This was confirmed below by Northern blot analysis.

AdiC does not affect the level or activity of AdiA. Computer-assisted analysis of AdiA indicated that the AdiA peptide sequence (QYPDTYANMGIHDLC) possessed good antigenicity and surface probability. As a result, this peptide was synthesized and used to raise antibody against AdiA (Genemed Synthesis, Inc). Anti-AdiA antibody was then used in

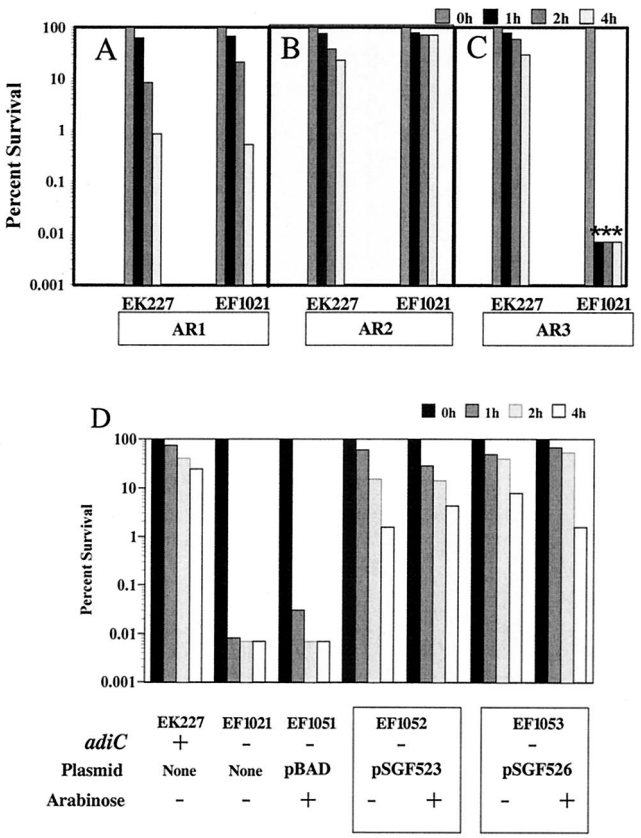

FIG. 3. An *adiC* mutant is specifically defective in arginine-dependent acid resistance. Acid resistance assays of EK227 (wild-type) and EF1021 (Δ*adi*C::Km). (A) AR 1. Cells were grown in LB-MES (pH 5.5) to stationary phase and challenged to EG medium (pH 2.5). (B) AR 2. Cells were grown in LB-glucose to stationary phase and challenged to EG medium (pH 2.0) in the presence of 0.7 mM glutamate. (C and D) AR 3. Cells were grown in BHIG to stationary phase (22 h) and challenged to EG medium (pH 2.5) with 1.5 mM arginine. Control cells grown in LB-MOPS (pH 8) (the control for AR 1) or cells challenged without glutamate or arginine (controls for AR 2 and 3, respectively) were completely inviable at 1 h post challenge (data not shown). (D) Cloned *E. coli adiC*$^+$ complements the Δ*adiC* arginine-dependent acid resistance defect. EF1051 (Δ*adiC*::Km/pBAD24), EF1052 (Δ*adiC*::Km/pSGF523), and EF1053 (Δ*adiC*::Km/pSGF526) were used. Values represent average survival after 0, 1, 2, and 4 h. All assays were performed in triplicate. Representative results are shown. Asterisks indicate that survival was below detection limits.

Western blots to investigate whether *adiC* mutations altered the levels of AdiA decarboxylase rather than transport. Figure 4 indicates that the *adiC* mutant and wild-type strains contained equivalent levels of AdiA. The *adiA* mutant control strain did not express any AdiA protein. Thus, AdiC does not affect the regulation of *adiA*.

However, even if AdiC did not affect *adiA* expression, it might still influence AdiA decarboxylase activity rather than arginine/agmatine transport. To address this possibility, we performed a direct measurement of internal ADC activity using cells solubilized with 0.1% Triton X-100. This treatment bypasses any requirement for membrane transport. The assays were conducted at pH 5, the reported optimal pH for inducible ADC (2). Figure 4 also illustrates that the *adiC* mutant and

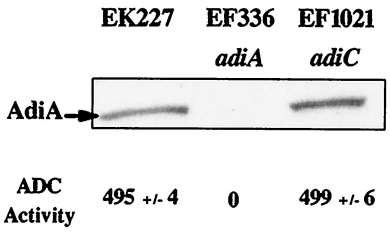

FIG. 4. An *adiC* mutation does not affect ADC levels or activity. Top, Western blot analysis of stationary-phase EK227 (wild-type strain), EF336 (*adiA*::mudJ), and EF1021(Δ*adiC*::Km). Whole-cell lysates prepared from BHIG-grown cells were separated on a 10% SDS-PAGE gel and blotted with anti-AdiA antibody. Bottom, ADC activity was measured at pH 5 in Triton X-100-solubilized cells. Units given are nanomolar of agmatine formed per hour per cell density unit (optical density at 600 nm).

wild-type strains exhibited equal levels of ADC activity. Only the *adiA* mutant failed to convert arginine to agmatine. Therefore, AdiC does not modify ADC activity or protein level.

The *adiC* mutant is defective in arginine/agmatine exchange. The abilities of wild-type and *adi*C and *adi*A mutant cells to take up arginine at pH 2.5, convert it to agmatine, and export the product were then assessed. Measurements of extracellular arginine and agmatine shown in Fig. 5A revealed that wild-type *E. coli* reciprocally linked a decrease in external arginine to an increase in external agmatine. Neither the *adiA* nor the *adiC* mutants could catalyze this exchange (Fig. 5B and C). Since *adiC* did not affect the synthesis or activity of AdiA as measured in solubilized cells (see above), the evidence supports a direct role for AdiC in the exchange of external arginine for internal agmatine.

The extracellular activation pH for arginine/agmatine exchange. The optimal pH for inducible ADC activity is pH 5.2, yet the arginine-dependent acid resistance system protects cells to an external pH of 2.5. Consequently, we asked what external pH value activates preexisting AdiA/AdiC in whole cells. EG media adjusted to pH 2.0, 2.5, 3.0, 4.0, 5.0, and 7.0, all containing 1 mM arginine, were used to determine the activating pH for this system. Cells in which the AdiA/AdiC system was induced were added to these media, and the conversion of extracellular arginine to agmatine was monitored. Figure 5D reveals that maximal external conversion of arginine to agmatine was observed, as predicted, at pH 2.5. There was almost no

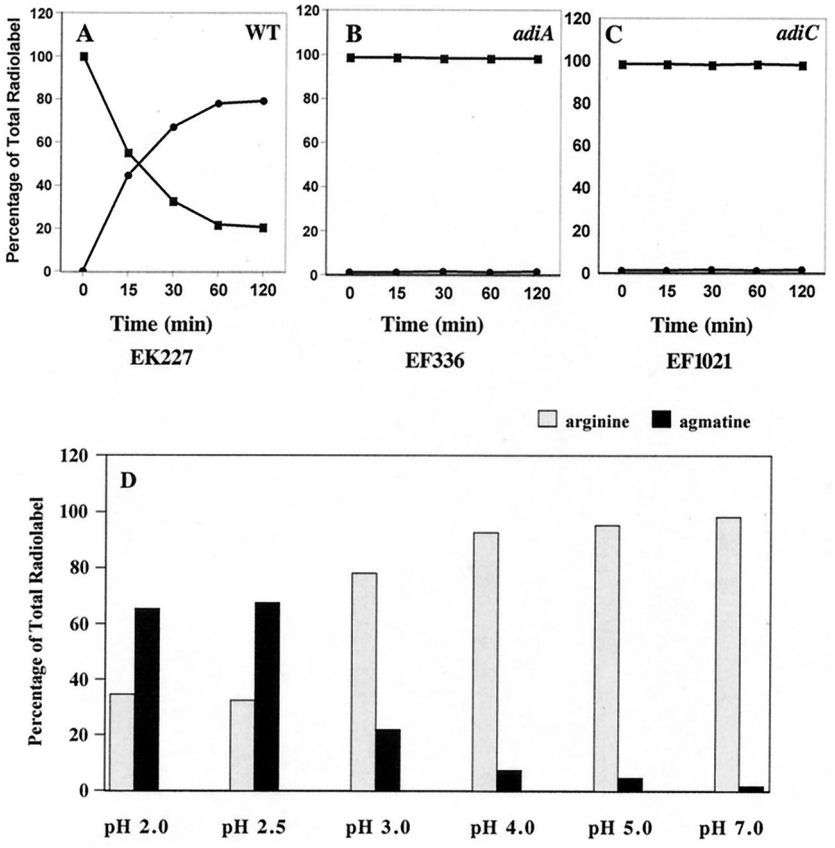

FIG. 5. Exchange of extracellular arginine for intracellular agmatine. Cells of strains EK227 (wild-type) (A), EF1021(Δ*adiC*::Km) (B), and EF336(*adiA*::Mu*dJ*) (C) were grown in BHIG for 22 h and adjusted to a cell density of 10^8/ml in EG medium at pH 2.5. The exchange of extracellular agmatine for arginine was measured by using 1 mM radiolabeled arginine. At various times, cell-free supernatants were collected from the cultures, adjusted to pH 7, spiked with unlabeled arginine and agmatine, and separated by paper chromatography. Spots corresponding to arginine and agmatine were cut into strips and counted for radioactivity. (D) Optimal pH for whole-cell exchange of arginine to agmatine by EK227.

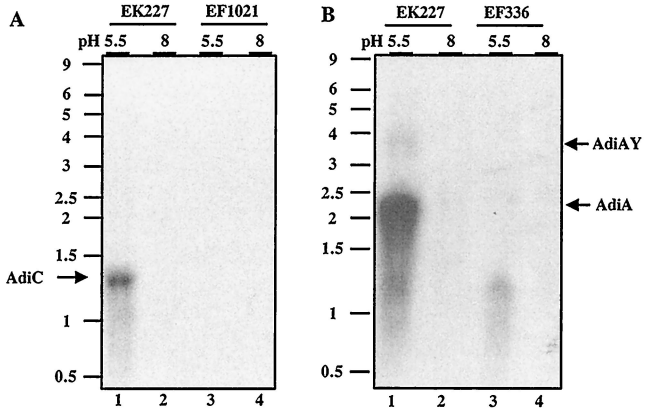

FIG. 6. Acid induction of *adiC* and *adiA*. Cells were grown anaerobically in ADC media to log phase (10^8 CFU/ml). Five micrograms of RNA was loaded per lane. Blots were probed either with *adiC* (A) or *adiA* (B). An RNA ladder (kilobases) is shown to the left of each panel. The 1.2-kb RNA band seen in panel B is unrelated to *adiA*, as it is seen in both the wild type and *adiA* mutants.

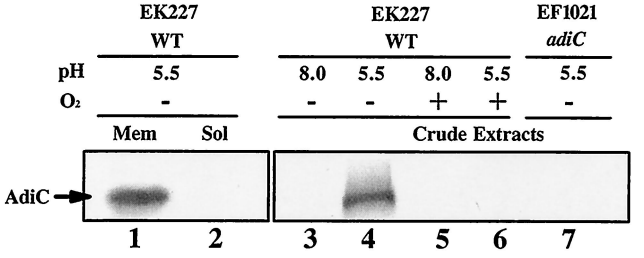

FIG. 7. Membrane location of the AdiC antiporter. Cells (EK227) were grown to exponential phase in ADC media with or without oxygen at the pH values indicated. Cells were harvested by centrifugation, sonicated, and separated into membrane (lane 1) and soluble (lane 2) fractions. Crude extracts were analyzed in lanes 3 through 7.

exchange when the medium pH was 3 or above. Thus, this decarboxylation and exchange system is most active under extreme acid conditions.

AdiC is acid inducible. The *adiA* gene encoding ADC is acid inducible (1). Northern blot analysis was performed to examine whether *adiC* was also acid induced and if it formed part of an operon with *adiA* and/or *adiY*. RNA extracts were probed for *adiA* and *adiC*. The results displayed in Fig. 6 indicate that *adiA* (panel B, lane 1 versus lane 2) and *adiC* (panel A, lanes 1 and 2) transcripts are acid induced in ADC cultures grown under anaerobic conditions. When blots were probed for *adiA*, both a major transcript comprised of *adiA* (2.2 kb) and a faint second band (3.2 kb) encompassing *adiAY* were evident.

Probing with *adiC* revealed only one transcript (1.3 kb). This mRNA was only large enough to encompass *adiC*. An *adiYC* transcript that was predicted, but never observed, in a previous study was not detected (18). Additional support for the existence of an *adiC*-specific promoter came from observing that an *adiY* mutant still exhibited arginine-dependent acid resistance (data not shown) and that *adiC*$^+$ cloned in two orientations relative to a plasmid-borne promoter still expressed AdiC (see above).

AdiC is a membrane protein. As an antiporter, AdiC should localize to the bacterial membrane. To demonstrate this, cells were grown anaerobically at pH 5.5 and 8 in ADC media and then separated into membrane and soluble fractions. The fractions were run on SDS-PAGE and probed with anti-AdiC antibody. Only the membrane fraction contained AdiC (Fig. 7). It is important to note that the SDS extracts of membrane preparations were not boiled for these studies. This is because AdiC tends to aggregate during boiling (data not shown). The observed AdiC monomer band ran at 34 kDa, faster than the calculated molecular weight of 46 kDa. However, this is not unusual for membrane proteins. Figure 7 also reveals that the synthesis of AdiC protein was regulated in a manner similar to that observed by Northern analysis (acid induced under anaerobic conditions).

DISCUSSION

E. coli produces an acid-inducible form of ADC, encoded by *adiA*, that contributes to the survival of this organism in extremely acidic environments (4, 10, 11). Mutants of *E. coli* that do not possess this enzyme fail to demonstrate arginine-dependent resistance to pH 2.5. Inducible amino acid decarboxylase systems typically possess, along with the amino acid decarboxylase, an antiporter that imports an extracellular amino acid substrate in exchange for the intracellular decarboxylation product. This exchange is needed to constantly replenish intracellular substrate and rid the cell of product. Prior to this report, the identity of the arginine:agmatine antiporter was unknown.

Twenty six Tn*10* insertion mutants specifically defective in arginine-dependent acid resistance were found to have mutations located within or upstream of *adiC* (*yjdE*), a gene predicted to encode an antiporter. Mutants lacking AdiC (YjdE) possessed normal levels of ADC activity, indicating that the gene does not regulate *adiA* expression or function. However, the mutants failed to convert extracellular arginine to agmatine, clearly supporting a role for AdiC as the requisite arginine:agmatine antiporter.

Northern blot analysis indicated that *adiC* is induced by growth under acidic conditions, but transcription appears to be independent of the *adiAY* promoter. Three transcripts were observed in the *adi* region, namely, (i) *adiAY* (minor), (ii) *adiA* (major), and (iii) *adiC*. These transcripts appear to result from two promoters, one before *adiA* and one preceding *adiC*. The finding that six of the Tn*10* insertions eliminating AdiC activity occurred upstream of the *adiC* open reading frame but not within *adiY* is consistent with *adiC* having an independent promoter (Fig. 2).

The data presented also revealed that maximal activity of the ADC-antiporter system in whole cells occurs at pH 2.5. At this pH, other transporters of arginine likely do not function, as evidenced by the failure of *adiA* and *adiC* mutants to remove arginine from the extracellular medium. Although *adiC* expression is induced by low pH, it is not known whether AdiC antiporter activity is directly under pH control or is constitutively active but used only at a pH where intracellular ADC is active. In either case, we predict that the arginine-dependent acid resistance system, to work efficiently at an external pH of 2.5, will maintain intracellular pH around 5, the optimum pH

for inducible ADC. Proton consumption would be maximal in this pH range for this system.

The *adi* locus also includes the gene *adiY* located between *adiA* and *adiC*. Earlier reports indicated that AdiY, a member of the XylS/AraC family of transcriptional regulators, was a positive regulator of *adiA* (18). In that study, overexpressing AdiY increased *adiA* transcription. However, in our screen for mutants defective in arginine-dependent acid resistance, no *adiY* mutants emerged. The targeted deletion of *adiY* also failed to alter arginine-dependent acid resistance under the conditions tested (data not shown). Another regulator controlling *adiA* is CysB (4, 15). CysB mutants are clearly defective in arginine-dependent acid resistance. Thus, as is the case for the glutamate decarboxylase (*gadA*/*BC*)-dependent acid resistance system, there may be multiple, and perhaps redundant, regulators for *adiA* and *adiC*. The AraC-like regulator GadX, for example, is needed to activate the *gadA*/*BC* genes only when cells are grown in complex media, not in minimal salts media (12). AdiY may fill an analogous role as a conditional regulator of the arginine-dependent system.

In sum, the inducible arginine:agmatine antiporter required for arginine-dependent acid resistance has been identified. This discovery will allow direct comparison between the arginine:agmatine antiporter and the putative glutamate:GABA antiporter employed by the glutamate-dependent acid resistance system. In addition, questions of pH control, exchange rates, and membrane configurations can now be addressed.

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