Efflux Permease CgAcr3-1 of Corynebacterium glutamicum Is an Arsenite-specific Antiporter*‡‡

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Background: CgAcr3-1 is an arsenite permease that catalyzes As(III) efflux from Corynebacterium glutamicum. Results: CgAcr3-1 is an As(III)-specific H+/As(OH)₃ antiporter coupled to the proton motive force.

Conclusion: CgAcr3-1 does not transport Sb(III). Mutagenesis indicates that two conserved residues, a cysteine and a glutamate, may be required for antiporter activity.

Significance: CgAcr3-1 is unusual in being able to transport As(III) but not Sb(III).

Resistance to arsenite (As(III)) by cells is generally accomplished by arsenite efflux permeases from Acr3 or ArsB unrelated families. We analyzed the function of three Acr3 proteins from Corynebacterium glutamicum, CgAcr3-1, CgAcr3-2, and CgAcr3-3. CgAcr3-1 conferred the highest level of As(III) resistance and accumulation in vivo. CgAcr3-1 was also the most active when everted membranes vesicles from Escherichia coli or C. glutamicum mutants were assayed for efflux with different energy sources. As(III) and antimonite (Sb(III)) resistance and accumulation studies using E. coli or C. glutamicum arsenite permease mutants clearly show that CgAcr3-1 is specific for As(III). In everted membrane vesicles expressing CgAcr3-1, dissipation of either the membrane potential or the pH gradient of the proton motive force did not prevent As(III) uptake, whereas dissipation of both components eliminated uptake. Further, a mutagenesis study of CgAcr3-1 suggested that a conserved cysteine and glutamate are involved in active transport. Therefore, we propose that CgAcr3-1 is an antiporter that catalyzes arsenite-proton exchange with residues Cys129 and Glu305 involved in efflux.

Arsenic is a toxic metalloid that is widespread in the environment from geochemical and anthropogenic sources. It ranks first on the Superfund List of Hazardous Substances (see the Centers for Disease Control Web site) due to its ubiquity and human health risk (1). Most organisms have mechanisms that detoxify As(III) to As(V) (2). Efflux of As(III) from cells is often the primary resistance mechanism and can be catalyzed by members of three families of unrelated As(III) transporters. The ATP-binding cassette transporters of the multidrug resistance-associated protein superfamily, including Mrp1 and Mrp2 in mammals (3), Ycf1p in yeast (4), and PgpA in Leishmania (5), generally sequester As(III) or antimonite (Sb(III)) in vacuoles or other cellular compartments or extruded them from cells in complex with glutathione or other thiols. The second arsenite efflux permease is ArsB, which, historically, was the first to be identified (6, 7). ArsB is widespread in bacteria and archaea and has a membrane topology with 12 transmembrane (TM) segments (8), which is similar to members of the major facilitator superfamily (9). The best studied metalloid transporter is the Escherichia coli ArsB protein, which is an antiporter that catalyzes the exchange of As(III) or Sb(III) for protons, coupling efflux to the electrochemical proton gradient (10). However, when the ATPase protein ArsA is expressed, hydrolysis of ATP provides the energy to sustain arsenite efflux. The ATP-coupled ArsAB efflux pump confers the highest level of As(III) and Sb(III) resistance (11).

The third arsenic resistance transporter is the arsenite permease Acr3, which is a member of the bile/arsenite/riboflavin transporter (BART) superfamily that includes members in bacteria, archaea, and fungi and is more widely distributed than ArsB (12). Acr3 transporters are smaller proteins than ArsB, and unfortunately, the literature is confused by the fact that many members of the Acr3 family are annotated as ArsB, although they exhibit no significant sequence similarity to ArsB. The first identified member of this family is encoded by the sigK operon of the skin (sigK intervening) element in the

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The abbreviations used are: As(V), arsenate; ICP-MS, inductively coupled plasma mass spectrometry; CCCP, carbonyl cyanide m-chlorophenylhydrazone; TM, transmembrane; As(III)/As(OH)₃, arsenite; Sb(III), antimonite; MMC, minimal medium for corynebacteria; Δp, proton motive force; BART, bile/arsenite/riboflavin transporter; TSB, tripticase soy broth; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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Acr3 Is an Arsenite Efflux Transporter in Corynebacteria

chromosome of Bacillus subtilis (13). The properties of a more distant Acr3 homologue from Shewanella oneidensis was examined recently (14); this protein confers "in vivo" resistance to As(V) but not As(III), and the purified protein binds only As(V), indicating that this protein is not a true Acr3 orthologue. Fungal members of this family include the ScAcr3p metalloid efflux protein from Saccharomyces cerevisiae, which was proposed to be selective for As(III) over Sb(III) (15). ScAcr3p has been suggested to transport Sb(III) as well (16), although other data do not agree with that conclusion (4). While this study was in progress, a recent report showed Sb(III) accumulation by yeast everted vesicles expressing Acr3 protein (17). Finally, an Acr3 homologue has recently been identified in plants and has been implicated in As(III) transport (18).

Corynebacterium glutamicum, a member of actinobacteria, is highly resistant to As(V) and As(III) due to the presence of two chromosomal ars operons (19). In the present study, we examined the properties of the arsenite permeases proteins from C. glutamicum (CgAcr3s). To date, most Acr3 transporters from bacteria and yeast have been reported to confer As(III) resistance (13, 15), whereas Acr3 from Synechocystis sp. (20) and from the yeast S. cerevisiae was reported to confer tolerance to both As(III) and Sb(III) (16). Our preliminary studies with expression of the acr3 genes of C. glutamicum (Cgacr3-1) and Alkaliphilus metallidigens (Amacr3-1) heterologously expressed in E. coli showed that they confer tolerance only to As(III).

To date, few mechanistic data are available for members of the Acr3 family. Only the S. cerevisiae Acr3p has been suggested to catalyze exchange of As(III) mediated by protons (17). Here, we demonstrate that CgAcr3-1 is a functional arsenite carrier in vitro. Everted membrane vesicles from E. coli and C. glutamicum containing CgAcr3-1 protein exhibited uptake of As(III) but not Sb(III). We propose here that CgAcr3-1 is an As(III)/H+ antiporter. We also show that CgAcr3-1 catalyzes uptake of As(III) into everted membrane vesicles by coupling to either the pH gradient or the membrane potential, both components of the electrochemical proton gradient. Based on the membrane topology of an Acr3 homologue, we performed site-directed mutagenesis of conserved hydrophilic residues in the predicted transmembrane domains of CgAcr3-1. Taking into account the results, we propose a model for the permease that is consistent with As(III) efflux data.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Growth Conditions, and Reagents—Bacterial strains and plasmids are described in supplemental Table S1. E. coli TOP10 was used as host strain for general cloning assays. E. coli cells were grown and maintained at 37 °C in Luria-Bertani (LB) medium. C. glutamicum cells were grown at 30 °C in complex medium triptase soy broth (TSB; Oxoid) or TSA (TSB containing 2% agar), or in minimal medium for corynebacteria (MMC). Growth in liquid cultures was routinely estimated from the absorbance at 600 nm (A600). Media were supplemented with kanamycin (12.5 μg/ml for C. glutamicum and 50 μg/ml for E. coli), ampicillin (100 μg/ml), or chloramphenicol (20 μg/ml) when necessary. Mobilizable plasmids were transferred by conjugation from E. coli S17-1 (donor strain) to the indicated recipient strains of C. glutamicum (21). All reagents were obtained from commercial sources. Most of the molecular techniques were performed using conventional protocols of general use (22) or more specific protocols for corynebacteria (23).

Construction of Plasmids and Strains—For expression studies, the three Cgacr3 genes from C. glutamicum (Cgacr3-1, Cgacr3-2, and Cgacr3-3), including the respective upstream hypothetical promoters, were PCR-amplified using the primer pairs acr3-1Fw/acr3-1Rv, acr3-2Fw/acr3-2Rv, and acr3-3Fw/acr3-3Rv (supplemental Table S2), respectively, and total DNA from C. glutamicum ATCC 13032 as template. The corresponding fragments of 1403 bp (Cgacr3-1), 1308 bp (Cgacr3-2), and 1197 bp (Cgacr3-3) were first digested by BamHI-HindIII and subcloned into the E. coli conjugative suicide plasmid pKarsIup (supplemental Table S1). After transformation of strain TOP10 and further selection, we obtained the recombinant suicide plasmids pKacr3-1, pKacr3-2, and pKacr3-3 (supplemental Table S1). The resulting vectors were independently used to transform either E. coli AW3110 (lacking the ars operon) or E. coli S17-1, which was used for plasmid mobilization to C. glutamicum 2Δars (supplemental Table S1).

Second, the 1403-bp (Cgacr3-1), 1308-bp (Cgacr3-2), and 1197-bp (Cgacr3-3) previously indicated fragments were Dral-digested (see the targets in the primer pair sequences; supplemental Table S2) and subsequently ligated to the mobilizable E. coli-corynebacteria shuttle vector pECM2 (EcoRV-digested) (supplemental Table S1); we obtained the recombinant plasmids pECacr3-1, pECacr3-2, and pECacr3-3, which were used to transform E. coli strain S17-1 (donor) and then mobilized to the corynebacteria strain 2Δars (recipient).

For immunoblot expression analysis, a Cgacr3-1 derivative gene that contains additional codons for six His residues was constructed, encoding the enzyme CgAcr3-1His. The Cgacr3-1 gene (including its own promoter sequence) was PCR-amplified using the primer pair acr3-1HisFw/acr3-1HisRv (supplemental Table S2). The PCR product carrying the His tag sequence (at its 3'-end) was first digested with BamHI and HindIII and subsequently cloned into plasmid pKarsIup (supplemental Table S1). The resulting expression vector was adequately characterized and named pKacr3-1His; this plasmid was used to transform either E. coli AW3110 or S17-1. Second, the same PCR product carrying Cgacr3-1His was Dral-digested and ligated into pECM2 (EcoRV-digested), yielding pECacr3-1His. This resulting vector was transfected by conjugation into C. glutamicum 2Δars.

Cgacr3-3 Gene Inactivation in C. glutamicum—An internal fragment of the Cgacr3-3 gene was PCR-amplified using the primer pair acr3-3intFw/acr3-3intRv (supplemental Table S2) and total DNA from C. glutamicum as template. The amplified 310-bp DNA fragment was purified, BamHI-HindIII-digested, and subcloned into the E. coli suicide vector pK18mob (supplemental Table S1). The resulting recombinant plasmid, pKacr3-3int, was used to transform S17-1 and mobilized to the recipient strain C. glutamicum 2Δars. To confirm the chromosomal Cgacr3-3 gene disruption, total DNA from the recipient strain was isolated and checked by PCR amplification analyses using the M13 universal primers (supplemental Table S2). Chromo-
somal integration of pKacr3–3int generated the triple acr3 mutant C. glutamicum 3acr3 (supplemental Table S1).

Oligonucleotide-directed Mutagenesis—Several codons for amino acid residues from Cgaer3-1 were individually replaced by alanine, phenylalanine, aspartic, or lysine residues with the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmids pKacr3-1 (wild-type Cgaer3-1) and pKacr3-1His (Cgaer3-1 carrying the sequence for the His tag at its 3′-end) were used as a template in PCR amplification with the primer pairs (supplemental Table S2) N-acr3K72A/C-acr3K72A, N-acr3C129A/C-acr3C129A, N-acr3R210A/C-acr3R210A, N-acr3T241A/C-acr3T241A, N-acr3E305A/C-acr3E305A, N-acr3E305D/C-acr3E305D, N-acr3E305F/C-acr3E305F, N-acr3E305K/C-acr3E305K, and N-acr3E322A/C-acr3E322A to generate plasmids pKacr3K72A, pKacr3C129A, pKacr3R210A, pKacr3T241A, pKacr3E305A, pKacr3E305D, pKacr3E305F, pKacr3E305K, and pKacr3E322A, respectively (supplemental Table S1), for pKacr3-1 derivatives and yielding plasmids pKacr3K72AHis, pKacr3C129AHis, pKacr3R210AHis, pKacr3T241AHis, pKacr3E305AHis, pKacr3E305DHis, pKacr3E305FHis, pKacr3E305KHis, and pKacr3E322AHis, respectively, for pKacr3-1His derivatives (supplemental Table S1). Plasmids containing the different gene mutations were used to transform either E. coli AW3110 or S17-1. In all cases, the mutations were verified by DNA sequencing.

Metalloid Resistance Assays—Resistance to As(III) and Sb(III) in E. coli and C. glutamicum was analyzed as follows. Strains of the two organisms were cultured in LB or TSB medium, respectively, and grown overnight in aerobic conditions and, when indicated, with the appropriate antibiotic. Then the cultures were diluted 100-fold into fresh LB medium (for E. coli) or TSB medium (for corynebacteria) containing the indicated concentrations of sodium arsenite or potassium antimonyl tartrate. The cultures were incubated at 37 or 30 °C, respectively, with vigorous shaking for an additional 24 h. Growth was estimated by A600. The resistance value for a strain to the indicated metalloid was defined as the metalloid concentration that causes at least 20% of the absorbance obtained for the same strain when growing in the same medium but lacking metalloid.

Metalloid Transport Assays in Whole Cells—For in vivo uptake assays, E. coli cells were grown in LB medium at 37 °C to a cell density (A600) of 1, and C. glutamicum cells were grown in MMC at 30 °C to a cell density (A600) of 2. The cells were harvested and suspended in a buffer solution of 75 mM Hepes-KOH, pH 7.5, 150 mM KCl, and 1 mM MgSO4 (buffer A) at a density of 10 or 6, respectively. To initiate the transport reaction, either sodium arsenite (0.1 mM for E. coli or 1 mM for C. glutamicum, final concentration) or potassium antimonyl tartrate (50 μM for E. coli or 40 μM for C. glutamicum, final concentration) was added to 1 ml of cell suspension. Portions of 100 μl from the cell suspension were withdrawn at the indicated times, filtered through nitrocellulose filters (0.45-μm pore diameter; Millipore), and washed twice at room temperature with 5 ml of buffer A. The filters were digested with 170 μl of 70% concentrated nitric acid (Sigma) at 70 °C and diluted with high pressure liquid chromatography (HPLC) grade water (Sigma) to produce a final 2% concentration of nitric acid. Arsenic and antimony were quantified by inductively coupled plasma mass spectroscopy (ICP-MS; ELAN-9000, PerkinElmer Life Sciences). Standard solutions were made in the range of 0.1–50 ppb in 2% nitric acid using arsenic and antimony standards (Ultra scientific, N. Kingstown, RI).

Metalloid Uptake Assays in Everted Membrane Vesicles—Cultures of E. coli and C. glutamicum were grown in 1 liter of LB or MMC at 37 °C, respectively, up to A600 = 2. E. coli everted membrane vesicles were prepared as follows. The culture was harvested, and then the pellet was suspended in buffer B (75 mM Hepes-KOH, pH 7.5, 150 mM KCl, 1 mM MgSO4, and 0.25 mM sucrose) and lysed by sonication at 30% amplitude (Fisher Sonic Dismembrator model 500). The lysate was treated with the serine protease inhibitor diisopropyl fluorophosphate (Sigma) and DNase I and then centrifuged at 20,000 × g for 20 min at 4 °C to remove unbroken cells and cell debris. The supernatant suspension was centrifuged at 150,000 × g for 1 h, and the pellet (containing the membrane fraction) was suspended in buffer B. The membrane vesicles were rapidly frozen in liquid nitrogen and stored at −80 °C.

To obtain C. glutamicum everted membranes vesicles, some additional steps were necessary. When the C. glutamicum culture reached the cell density (A600) of 2, 0.3 unit/ml of penicillin G was added for 5 h. The cells were harvested at 20,000 × g for 10 min. The pellet was suspended in 1/100 of the initial volume in buffer C (MMC diluted by half with a solution containing 0.25 M sucrose and 10 mM MgSO4) and incubated with 2 mg/ml lysozyme overnight at 30 °C without shaking. The protoplasts were centrifuged at 10,000 × g for 10 min at 4 °C and suspended in buffer B. The final steps were the same as for E. coli everted membrane vesicles. For uptake assays in which the everted membranes were treated with thiol-modifying reagents, whole E. coli cells and C. glutamicum protoplasts were washed with buffer B without MgSO4, equilibrated at 37 °C, and suspended in buffer B containing 2 mM EDTA. After 10 min, MgSO4 was added to a final concentration of 10 mM. The EDTA-treated E. coli or C. glutamicum cells were harvested, the pellet was suspended in buffer B, and the remaining steps were the same as described for preparation of E. coli everted membranes.

Vesicles transport assays were performed in transport buffer D (75 mM Hepes-KOH, pH 7.5, 0.25 M sucrose, 1 mM MgSO4, and 150 mM K2SO4). The reaction mixture contained 1 mg/ml membrane proteins, 100 μM sodium arsenite (or 40 μM potassium antimoniyl tartrate for E. coli or 4 μM potassium antimonyl tartrate for C. glutamicum) in a final volume of 0.6 ml of buffer D; the reaction was started by the addition of 5 mM NADH (final concentration) or other energy sources, as indicated. The permutant anion SCN− (as KSCN), the weak base NH4+ (as NH4Cl), or the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added, as indicated. Portions of 100 μl were withdrawn at the indicated times, filtered through nitrocellulose filters (0.45-μm pore size), and washed twice with 5 ml of buffer D. The arsenic or antimony content was determined by ICP-MS.

Fluorescence Analysis of pH Gradient (ΔpH) and Membrane Potential (ΔΨ)—Formation of ΔpH and ΔΨ in everted membranes vesicles was estimated from the quenching of quinacrine
orange and oxonol V fluorescence, respectively (10). The reaction mixture consisted of 20 mM Hepes-KOH, pH 7.5, 2.5 mM MgSO$_4$, and 0.13 mg of membrane proteins containing 0.1 m KCl and 2 mM quinacrine orange for measuring $\Delta$P/\$h$ in a final volume of 2 ml. Quenching was initiated at room temperature by the addition of a final concentration of 5 mM NADH (or a different energy source when indicated). Fluorescence was measured in stirred cuvettes with a spectrophotometer (Photon Technology International, Inc.) with excitation at 492 nm and emission at 527 nm for quinacrine orange (Sigma) and excitation at 589 nm and emission at 616 nm for oxonol V (Sigma).

Expression and Immunological Detection of CgAcr3 Proteins—Membrane proteins were isolated as described elsewhere (24) with some modifications. Briefly, E. coli or C. glutamicum cells were suspended in buffer B and lysed by “FastPrepTM FP120” (Thermo BIO101) using glass beads of 0.15–0.212 mm (or a different energy source when indicated). Fluorescence was measured in stirred cuvettes with a spectrophotometer (Photon Technology International, Inc.) with excitation at 492 nm and emission at 616 nm for oxonol V (Sigma).

RESULTS

C. glutamicum CgAcr3-1 and CgAcr3-2 Are Involved in Arsenite Tolerance—Previously, we constructed three C. glutamicum mutant strains in which each Cgacr3 gene was disrupted (26). The observed As(III) resistance levels obtained for the mutants indicate that Cgacr3-1 and Cgacr3-2 are involved in arsenite resistance in C. glutamicum, whereas Cgacr3-3 is not (Fig. 1A). This As(III) resistance role was corroborated when the double Cgacr3 gene-disrupted mutant C. glutamicum 2Acr3 exhibited extreme As(III) sensitivity (Fig. 1A). Equivalent As(III) resistance levels were obtained for the As(III)-hypersensitive strain C. glutamicum 2Δars, which lacks both ars1 and ars2 operons (Fig. 1A) (27). We used C. glutamicum 2Δars as the recipient strain for homologous complementation analysis of the CgAcr3 proteins, whereas E. coli AW3110 (where the chromosomal arsRBC operon was replaced by the cat gene) was used for CgAcr3 heterologous complementation analysis. To ascertain whether the orphan gene Cgacr3-3 was functional, we performed additional gene disruption analysis by transferring the mobilizable suicide plasmid pKacr3-3int to the C. glutamicum 2Δars background, obtaining the strain C. glutamicum 3Acr3 (supplemental Table S1). The resistance analyses data (Fig. 1A) show that CgAcr3-3 does not contribute to As(III) resistance in C. glutamicum.

Heterologous and homologous complementation analyses of Cgacr3s expressed under their own promoters were performed either in E. coli AW3110 (corynebacterial promoters are frequently functional in E. coli) (26, 28), using the pKars1up derivative vectors pKacr3-1, pKacr3-2, and pKacr3-3, or in C. glutamicum 2Δars, in the latter case using integrative suicide (pKacr3-1, pKacr3-2, and pKacr3-3) or bifunctional (pEcacr3-1, pEacr3-2, and pEcacr3-3) vectors. Integrative pKars1up derivatives were incorporated into a 0.5-kb chromosomal region located at the 5’-upstream region of the ars1 C. glutamicum operon by means of an identical 0.5-kb fragment present in all of the pKars1up derivatives. The As(III) resistance and accumulation values reached by C. glutamicum 2Δars and E. coli AW3110 strains after homologous or heterologous Cgacr3 complementation analyses were in full agreement with our previous results (Fig. 1, B and C). Overall, we can conclude that CgAcr3-1 conferred almost 80% of arsenite permease activity even when it was heterologously expressed in E. coli, and CgAcr3-2 was responsible for the remaining 20% of permease activity (Fig. 1, B and C). No activity was apparently attributable to CgAcr3-3 when the corresponding C. glutamicum 2Δars transconjugants or E. coli AW3110 transformant strains (in both cases containing Cgacr3-3) were used for As(III) resistance or accumulation analysis (Fig. 1, B and C).

Immunological detection of the CgAcr3-1 protein was accomplished by Western analysis using antibodies against the CgAcr3-1His tag-fused protein or by specific CgAcr3-1 anti-peptide antibodies. Expression of CgAcr3-1 in C. glutamicum (Fig. 1B, inset) or in E. coli (Fig. 1C, inset) mutant strains was observed by Western analysis. The results indicate that CgAcr3-1 is adequately expressed and detected using either antibody. Moreover, CgAcr3-2 was detected at levels apparently equivalent to those of CgAcr3-1 when specific CgAcr3-1 antibodies were used (cross-reaction; insets of Fig. 1, B and C); this was not the case for the predicted CgAcr3-3 protein. No significant differences were observed in Western analysis when CgAcr3-1 was expressed in C. glutamicum from pKars1up derivative plasmids (as chromosomal integrated monocyop) or from the bifunctional pECM2 derivatives (as multicopy) (Fig. 1B).

A comparative analysis of Acr3s proteins, including the three from C. glutamicum was performed (supplemental Fig. S1).
**FIGURE 1.** CgAcr3-1 is the primary arsenite permease in *C. glutamicum*. In each panel, growth ($A_600$) of wild-type and mutant strains was assayed at the indicated As(III) concentrations, and resistance (■ columns) was determined as indicated under “Experimental Procedures.” Membranes from *C. glutamicum* or *E. coli* were prepared as described under “Experimental Procedures.” Error bars, S.D. from three assays. A, As(III) resistance assays were performed in *C. glutamicum* strains with disrupted or deleted Cgacr3 genes: wild type strain containing the three CgAcr3 permeases (RES167); disrupted Cgacr3-1 gene (Acr3-1); disrupted Cgacr3-2 gene (Acr3-2); disrupted Cgacr3-3 gene (Acr3-3); double Cgacr3-1 and Cgacr3-2 gene disruption (strain 2Acr3); mutant with the two ars operons deleted (2Δars); and triple mutant where Cgacr3-1 and Cgacr3-2 genes are deleted and Cgacr3-3 gene is disrupted (strain 3Acr3). B, *C. glutamicum* 2Δars (host strain used as control) and transconjugants containing gene Cgacr3-1, Cgacr3-2, or Cgacr3-3 in two different kinds of vectors (the integrative pKars1up (generating pKacr3-1, pKacr3-2, or pKacr3-3) and the bifunctional pECM2 (generating pECacr3-1, pECacr3-2, or pECacr3-3)) were assayed for CgAcr3 protein expression by immunoblot using antibodies against CgAcr3-1 (inset), As(III) resistance (■ columns; left axis), and transport of As(III) (□ columns; right axis). C, cells of *E. coli* AW3110 (host strain) containing Cgacr3-1, Cgacr3-2, or Cgacr3-3 in vector pKars1up (pKacr3-1, pKacr3-2, or pKacr3-3) were assayed for CgAcr3s protein expression by immunoblot using antibodies against CgAcr3-1 (inset), As(III) resistance (■ columns; left axis), and transport of As(III) (□ columns; right axis). *E. coli* AW3110 (strain lacking the ars operon) and *E. coli* W3110 (wild type) were used as controls.
CgAcr3-1 (370 amino acids) and CgAcr3-2 (366 amino acids) are 72% identical to each other, yet their expression produces very different activity in vivo. The two permeases are 18–20% identical with CgAcr3-3 (287 amino acids), which seems to have only seven or eight TM domains, by using different topological analysis programs: (i) S-TMHMM server version 2.0, (ii) TopPred2, and (iii) SOSUI. The possible absence of several TM domains might explain its apparent lack of activity. Notably, CgAcr3-1, CgAcr3-2, and CgAcr3-3 each have the conserved cysteine (Cys129 in CgAcr3-1) that is found in all Acr3 homologues examined to date (supplemental Fig. S1), although each has additional cysteines that are not conserved in other homologues. A putative topology for CgAcr3-1 (Fig. 2) was deduced by protein sequence alignment with the A. metalliredigens Acr3 protein. Because CgAcr3-1 appears to be the major arsenite permease in C. glutamicum, further analyses were accomplished using only Cgacr3-1 expressed behind its own promoter and subcloned in pKars1up.

**CgAcr3-1-catalyzed Arsenite Efflux Is Coupled to Proton Motive Force (Δp)—**Decreased As(III) accumulation in cells of either E. coli or C. glutamicum expressing Cgacr3-1 compared with those lacking the gene reflects active extrusion by CgAcr3-1 (Fig. 1, B and C). The energy source used by CgAcr3-1 for As(III) efflux has not been identified. To address this, we first examined intact cells of C. glutamicum and second examined everted membrane vesicles from E. coli and C. glutamicum. Whole cells of C. glutamicum RES167 (Fig. 3A), C. glutamicum 2Δars (Fig. 3B), and C. glutamicum 2Δars containing pKacr3-1 (with the Cgacr3-1 gene; Fig. 3C) were assayed in the presence of As(III) with or without carbonyl cyanide m-chlorophenylhydrazone (CCCP), which dissipates the entire proton motive force (Δp). Little As(III) was accumulated in either C. glutamicum RES167 or C. glutamicum 2Δars containing pKacr3-1 strains, presumably as the result of efflux catalyzed by CgAcr3-1 (Fig. 3, A and C). However, in the presence of the CCCP, As(III) accumulation increased in both strains, reflecting uncoupling of efflux from the cells. The mutant C. glutamicum 2Δars (lacking both CgAcr3-1 and CgAcr3-2 permeases) accumulated equivalent amounts of As(III) in the presence or absence of CCCP (Fig. 3B), indicating little efflux in those strains. These results demonstrate that CgAcr3-1 is coupled to the proton motive force in whole cells. To examine the energy coupling of CgAcr3-1 in more detail, everted membrane vesicles from E. coli AW3110 or C. glutamicum 2Δars expressing Cgacr3-1 were prepared. Respiration of NADH in everted membrane vesicles creates an electrochemical proton gradient (Δp), acid and positive inside, and drives accumulation of As(III) in vesicles from both bacterial strains (Fig. 4, A and B). No uptake was observed (i) in vesicles from cells lacking Cgacr3-1, (ii) when membrane vesicles were obtained by other cell lysis methods (see “Discussion”), or (iii) when NADH was absent (see Fig. 5, A and B). No respiration-driven As(III) uptake was observed with the addition of different potential respiratory substrates, such as lithium-D-lactate, proline, succinate, acetate, ascorbate, and phenazine methosulfate (Table 1). Accumulation of As(III) in vesicles required both CgAcr3-1 and
NADH, and the addition of CCCP produced a rapid reversal of As(III) accumulation (Fig. 4, A and B). NADH oxidation by everted membranes vesicles from both C. glutamicum and E. coli generated sufficient $\Delta^H$ to incorporate As(III) inside of everted membranes. $\Delta^H$ consists of an electrical component ($\Delta \phi$), estimated from the quenching of oxonol V fluorescence, and a chemical component ($\Delta pH$), estimated from the quenching of quinacrine orange fluorescence (see supplemental Fig. S2). Other respiratory substrates did not generate sufficient $\Delta^H$, although the addition of ascorbate plus phenazine methosulfate was partially active (30% compared with NADH) (Table 1). These results clearly show that As(III) transport catalyzed by CgAcr3-1 is coupled to the electrochemical proton gradient established basically by NADH respiration.

Acr3 Is an Arsenite Efflux Transporter in Corynebacteria

CgAcr3-1 Catalyzes As(OH)₃/$H^+$ Exchange—Considering the uncoupling effect of CCCP on NADH-driven As(III) uptake in everted membrane vesicles, we might propose that CgAcr3-1 is a uniporter for the arsenite anion AsO₂$/H^+$, as was originally proposed for ArsB (29). However, ArsB was subsequently shown to transport the neutral species As(OH)₃ in exchange for protons (10). We considered the possibility that Acr3s might also transport the neutral As(OH)₃ into the acid and positive interior of the everted vesicles by exchange with a cation or proton. Dependence on a specific cation was investigated in E. coli and C. glutamicum everted membrane vesicles with an artificial $\Delta pH$ gradient, using two methods as described previously (30). First, an artificial potassium gradient was generated by nigericin-mediated K$/H^+$ exchange in K⁺-loaded membrane vesicles, which created a $\Delta^pH$. Second, an artificial sodium gradient was created by loading the membranes with sodium and then by dilution into sodium-free assay buffer. However, neither provided a driving force for uptake of As(III) in everted membrane vesicles (supplemental Fig. S3).

To examine the possibility that CgAcr3-1 is an electrophoretic As(III)/$H^+$ antiporter, the effect of either a permeant anion (thiocyanate, SCN⁻) or a permeant weak base (ammonium, NH₄⁺) on As(III) uptake was assayed in everted membrane vesicles from both E. coli and C. glutamicum expressing CgAcr3-1 (Fig. 4, A and B). When both SCN⁻ and NH₄⁺ were added 5 minutes after the accumulated As(III) was released (Fig. 4, A and B), uncoupling As(III) transport as effectively as
CCCP. However, neither SCN− alone (which dissipates the positive interior Δψ but does not dissipate ΔpH) nor NH4+ alone (which dissipates the acid interior ΔpH but does not dissipate Δψ) had a significant effect on NADH-driven accumulation of As(III) in everted vesicles (Fig. 4). These results indicate that either individual component of the electrochemical proton gradient, Δψ or ΔpH, is capable of energizing As(III) transport via CgAcr3-1. Because dissipation of only one of the Δp components (Δψ or ΔpH) does not inhibit As(III) transport, CgAcr3-1 cannot be an electrophoretic anion uniporter but is more likely an electrophoretic metalloid-proton exchanger with As(OH)3/H+ antiporter activity.

### Substrate Dependence of As(III) Uptake

As(III) uptake assays in everted membranes from *C. glutamicum* 2Δars with or without plasmid pKacr3-1 were performed. We detected hyperbolic saturation kinetics with an apparent $K_m$ of 0.03 mM and a $V_{max}$ of ~280 nmol/mg membrane protein/min (supplemental Fig. S4), a clearly different observation compared with the apparent $K_m$ of 0.14 mM for As(III) efflux by the ArsB pump of *E. coli* (10).

| Addition                                    | As(III) uptakea | Percent |
|---------------------------------------------|-----------------|---------|
| 5 mM NADH                                   | 3.76            | 100     |
| 20 mM ascorbate + 3 mM phenazine methosulfate | 1.16            | 31      |
| 20 mM sodium succinate                      | 0.06            | 1       |
| 20 mM proline                               | 0.05            | 2       |
| 20 mM sodium acetate                        | 0.07            | 2       |

a Arsenite uptake in everted membrane vesicles from *C. glutamicum* expressing Cgacr3-1.

### Table 1

**Effect of different energy sources on arsenite transport via CgAcr3-1 in *C. glutamicum* 2Δars everted membrane vesicles**

**CgAcr3-1 Is Specific for Arsenite and Does Not Transport Antimonite**—Despite the chemical similarity of As(III) and Sb(III), we had previously shown that heterologous expression of Cgacr3-1 in *E. coli* AW3110 cells conferred As(III) resistance and increased efflux of As(III), but this was not the case for Sb(III) (24). However, transport of Sb(III) by CgAcr3-1 had not been assayed in *C. glutamicum* or in everted membrane vesicles from either *E. coli* or *C. glutamicum*. We assayed Sb(III) uptake in three strains of *C. glutamicum*, RES167, 2Δars, and 2Δars, with pKacr3-1 integrated in the chromosome (as monocopy). All of the strains exhibited equivalent sensitivity (20 μM) and amount of the Sb(III) accumulated (0.12 nmol/mg biomass). Thus, there was no correlation of the Sb(III) phenotype (resistance or accumulation) in the presence or absence of CgAcr3-1, CgAcr3-2, or CgAcr3-3. These results demonstrate that none of the CgAcr3 permeases catalyzes Sb(III) efflux from cells of *C. glutamicum*. To examine this question in more depth, we assayed As(III) and Sb(III) uptake with everted membrane vesicles from *E. coli* and *C. glutamicum*. Everted membranes from *E. coli* AW3110 or *C. glutamicum*...
**Acr3 Is an Arsenite Efflux Transporter in Corynebacteria**

**TABLE 2**

| Addition                  | As(III) uptake* |
|---------------------------|-----------------|
|                           | umol/mg membrane protein/min | Percent |
| None                      | 3.76            | 100 |
| 10 mM Na$_2$HASO$_4$      | 3.48            | 92  |
| 10 mM SbCl$_3$            | 3.38            | 90  |
| 10 mM potassium antimonyl tartrate | 3.33        | 89  |
| 10 mM Na$_2$SO$_3$        | 2.69            | 71  |
| 10 mM NaNO$_2$            | 2.62            | 96  |
| 10 mM Na$_2$S$_{2}$O$_3$  | 2.70            | 72  |
| 10 mM K$_2$PO$_4$         | 3.02            | 80  |
| 10 mM HBO$_3$             | 2.67            | 70  |

*Arsenate uptake was assayed in everted membrane vesicles from *C. glutamicum* expressing the Cgacr3-1 gene in the presence of NADH.

**tamicum** 2Δars containing pKacr3-1 accumulated 5.5 (Fig. 5A) or 4.0 (Fig. 5B) nmol of arsenic/mg of membrane protein, whereas the same vesicles accumulated only 0.20 (Fig. 5C) or 0.03 (Fig. 5D) nmol of antimony/mg of membrane protein when NADH was used as a respiratory substrate. Moreover, vesicles made from cells with or without CgAcr3-1 showed the same low level of Sb(III) uptake. We also tested the effect of different oxyanions on As(III) uptake via CgAcr3-1 in *C. glutamicum* everted membrane vesicles (Table 2). None of the oxyanions tested, including the sodium or potassium salts of AsO$_4^{3-}$, PO$_4^{3-}$, NO$_3^-$, NO$_2^-$, SO$_4^{2-}$, BO$_3^-$, SbCl$_5^-$ or potassium antimonyl tartrate, had a significant effect on As(III) uptake. Our results show that CgAcr3-1 is a specific As(III) permease.

Cys$_{129}$ and Glu$_{305}$ are involved in As(III) Extrusion by CgAcr3-1—Structure-function relationships in CgAcr3-1 were studied by site-directed mutagenesis. There are no protein structural data for any Acr3 or other related BART proteins known. Therefore, we used an experimentally determined transmembrane topology of Acr3 from *A. metallireducens* to generate a structural alignment of both AmAcr3 and CgAcr3-1 permeases and a putative CgAcr3-1 topological map (Fig. 2). The participation of six conserved residues in the activity of CgAcr3-1 was examined by site-directed mutagenesis: Lys$_{72}$, Arg$_{210}$, Thr$_{241}$, Glu$_{305}$, and Glu$_{332}$ (Fig. 2, black triangles, and supplemental Fig. S1, vertical arrows), localized respectively in transmembrane segments TM2, TM4, TM6, TM7, TM9, and TM10 (Fig. 2). Previously, the role of Cys$_{129}$ from CgAcr3-1 was studied by expression in *E. coli* AW3110 (24). We analyzed the effect of site-directed mutagenesis to alanine of these six residues in *in vivo* and *in vitro* analyses. For *in vivo* assays, the six CgAcr3-1 mutants were expressed by pKars1up derivative vectors (under the Cgacr3-1 promoter) in strains *E. coli* AW3110 and *C. glutamicum* 2Δars, and characterized phenotypically for As(III) resistance in liquid media (Fig. 6A). *E. coli* and *C. glutamicum* cells expressing wild-type CgAcr3-1 grew in medium containing 8 mM As(III), whereas cells without the *ars* operons were sensitive at ~0.5 mM As(III) (Fig. 6A). Replacement of Lys$_{72}$, Arg$_{210}$, Thr$_{241}$, and Glu$_{332}$ to alanine (K72A, R210A, T241A, and E332A, respectively) did not significantly alter As(III) resistance compared with the wild type CgAcr3-1 protein. In contrast, Cys$_{129}$ (C129A) and Glu$_{305}$ (E305A) substitutions eliminated resistance of strains to As(III) (Fig. 6A). For *in vitro* assays, As(III) uptake in *E. coli* everted membranes vesicles expressing the six mutants was analyzed (Fig. 6B). Only C129A and E305A substitutions decreased As(III) transport, whereas replacements of K72A, R210A, T241A, and E332A had little effect on uptake (Fig. 6B).

The expression levels of wild-type and mutant CgAcr3-1s in *E. coli* and *C. glutamicum* cells were estimated by immunoblot analysis using antiserum against either wild-type CgAcr3-1 (Fig. 6A) or anti-His antibodies (data not shown). For His tag detection, the six CgAcr3-1 variants were constructed and expressed in pKacr3-1His derivative vectors in *E. coli* AW3110 and *C. glutamicum* 2Δars strains. The original and variant proteins were expressed at equivalent levels (Fig. 6A, immunoblot inset). Thus, these six Cgacr3-1 mutations do not appear to affect synthesis and/or overall folding of the CgAcr3-1 protein. These results are consistent with residues Cys$_{129}$ and Glu$_{305}$ but not Lys$_{72}$, Arg$_{210}$, Thr$_{241}$, or Glu$_{332}$, being involved in As(III) translocation by CgAcr3-1.

The role of Cys$_{129}$ and Glu$_{305}$ was examined in more detail. Cys$_{129}$ was necessary for As(III) transport by CgAcr3-1 in *E. coli* cells (24); in the present work, As(III) uptake was assayed in *E. coli* and *C. glutamicum* everted vesicles expressing wild type CgAcr3-1 treated with different thiol reagents (Fig. 7, A and B). Everted membrane vesicles from *E. coli* or *C. glutamicum* were treated either with the non-permeable membrane reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) or with the membrane-permeable thiol reagent N-ethylmaleimide or iodoaceticamide. In either type of vesicles, As(III) uptake was inhibited to background levels by the permeable reagent N-ethylmaleimide or iodoaceticamide (Fig. 7). In contrast, As(III) uptake in *E. coli* and *C. glutamicum* everted membrane vesicles was not affected by DTNB (Fig. 7). These results are consistent with Cys$_{129}$ being located in a transmembrane segment, as previously reported for CgAcr3-1 expressed in *E. coli* cells and with it playing a key role in As(III) translocation (24). To examine the role of the amino acid Glu$_{305}$ on As(III) translocation in more detail, Glu$_{305}$ was replaced by residues of different sizes and hydrophobicity by site-directed mutagenesis (see primers from supplemental Table S2), using as template the vectors pKacr3-1 or pKacr3-1His (the latter for expression level analysis). Substitutions of Glu$_{305}$ with a smaller residue (E305A), a larger residue (E305F), a positively charged residue (E305K), or a negatively charged residue (E305D) all decreased As(III) uptake in everted vesicles from *E. coli* AW3110 cells expressing Cgacr3-1 (Fig. S4). Consistent results were also obtained for As(III) accumulation using whole *E. coli* cells (Fig. 8B) and for arsenite resistance of the corresponding cultures (Fig. 8C). The expression levels of Glu$_{305}$ variants were similar to the wild type (Fig. 8A, inset), verifying that these replacements do not affect synthesis or overall folding of CgAcr3-1.
results are consistent with a role for both Cys$^{129}$ and Glu$^{305}$ in As(III) translocation by CgAcr3-1.

**DISCUSSION**

In *C. glutamicum*, three arsenite permease genes are located at different positions in the chromosome. Two of them, Cgacr3-1 and Cgacr3-2, encode active arsenite efflux proteins, whereas Cgacr3-3 does not seem to be functional. The presence of several gene copies for bacterial As(III) detoxification is not an infrequent occurrence (31). It allows organisms, such as the saprophytic *C. glutamicum*, to respond to high concentrations of environmental As(III). CgAcr3-1 confers 80% of the resist-
Acr3 Is an Arsenite Efflux Transporter in Corynebacteria

A. coli

E. coli

As(III) uptake (nmol/mg membrane protein)

+ plasmid/ - addition
+ plasmid/ + DTNB
+ plasmid/ + NEM
+ plasmid/ + IAA
+ plasmid/ addition

B. C. glutamicum

As(III) uptake (nmol/mg membrane protein)

+ plasmid/ - addition
+ plasmid/ + DTNB
+ plasmid/ + NEM
+ plasmid/ + IAA
+ plasmid/ + cation
+ plasmid/ addition

FIGURE 7. Effect of thiol reagents on arsenite uptake by CgAcr3-1 in everted vesicles. As(III) uptake was assayed as described in the legend of Fig. 4. Shown are everted membrane vesicles from E. coli AW3110 (A) or C. glutamicum 2Aars (B) without plasmid (●) or expressing pAcr3-1 (open symbols) and with the following additions: no addition (● and ○), 1 mM N-ethylmaleimide (NEM) (◇), 1 mM iodoacetamide (IAA) (△), and 1 mM DTNB (□). The compounds were added just prior to initiate the reaction.

ance, and CgAcr3-2 carries out the remaining 20%. It is not clear why CgAcr3-1 and CgAcr3-2 confer different levels of As(III) resistance because they are very similar to each other (72% sequence identity) (26). The results of immunoblots (Fig. 1, B and C) suggest that they are expressed at roughly equivalent levels, assuming that they react similarly with the CgAcr3-1 polyclonal antibodies. CgAcr3-3, on the other hand, lacks two transmembrane domains, which is also observed in other homologues. This may explain why CgAcr3-3 is not an active As(III) efflux protein. In addition, CgAcr3-3 has a lower sequence similarity with other Acr3 proteins (see alignments in supplemental Fig. S1), it is not regulated by the sequence similarity with other Acr3 proteins (see alignments in Fig. 3), suggesting that CgAcr3-1 is coupled to the proton motive force (Δp). However, it is still possible that CgAcr3-1 is coupled to chemical energy, either as ATP or some other high energy compound, which is later depleted as the proton motive force runs down. The uncoupler effect also does not exclude coupling to other gradients, such as sodium or potassium.

The As(III) uptake into everted membrane vesicles is more conclusive because the energy sources are more defined than in intact cells (10), so everted membrane vesicles from C. glutamicum cells expressing CgAcr3-1 were prepared. Because whole cells extrude As(III), everted membrane vesicles would be expected to accumulate it (10). With the protocol for obtaining everted membranes from E. coli expressing ArsB (10, 29), we were not able to incorporate As(III) in membranes when the protein AmAcr3 or CgAcr3-1 was expressed either in E. coli or C. glutamicum. Therefore, we prepared everted membrane vesicles from C. glutamicum cells after disrupting by sonication, using NADH as an energy source. Here, As(III) accumulated via CgAcr3-1 (see Fig. 5). In both strains of E. coli and C. glutamicum everted vesicles, As(III) uptake was sensitive to compounds that decreased the proton motive force (Fig. 4). These results are consistent with As(III) translocation by CgAcr3-1 coupled to the proton motive force. However, this does not define the mechanism of energy coupling. ArsB from E. coli is an antiporter in which the neutral As(OH)3− is exchanged for H+ driven by proton motive force (10). For CgAcr3-1 an equivalent role is possible, although another possibility is that it is a uniporter, where cellular efflux of the arsenite anion As(OH)3O− would be driven by the membrane potential, positive outside (24). As(OH)3− is a neutral molecule at cytosolic pH (10), so efflux mechanisms in which a neutral substrate is coupled to the electrochemical proton gradient must be considered. Extrusion of a neutral molecule from cells to the acid and positive exterior could be accomplished by exchange with a cation. Because there was not apparent requirement for either sodium or potassium ion (supplemental Fig. S3), it was reasonable to consider exchange with protons. To test this hypothesis, we applied an electrochemical proton gradient as a membrane potential or as a pH gradient. Either was capable of supporting As(III) uptake into everted membrane vesicles. These results were inconsistent with either a uniporter for a neutral soluble molecule, which would catalyze only facilitated diffusion, or a uniporter for an anion, which would be able to couple only to the membrane potential and not the pH gradient. The most reasonable explanation for these results is that CgAcr3-1 is an As(III)-proton antiporter.

ArsB metalloid extrusion permeases clearly catalyze efflux of either As(III) or Sb(III), with higher affinity for Sb(III) (10). The situation with members of the Acr3 family is not as clear. In S. cerevisiae and Synechocystis, Acr3 proteins are involved in extrusion and resistance to both As(III) and Sb(III) (17, 20). In both organisms, aquaglyceroporins facilitate rapid uptake of logues lack a recognizable nucleotide binding domain, and therefore it is reasonable to assume that CgAcr3 proteins are secondary efflux carriers for As(III). How is CgAcr3-1 coupled to metabolic energy? The addition of the Δp uncoupler agent CCCP to C. glutamicum cells containing only the arsenite permease CgAcr3-1 prevented As(III) efflux (Fig. 3), suggesting that CgAcr3-1 is coupled to the proton motive force (Δp). However, it is still possible that CgAcr3-1 is coupled to chemical energy, either as ATP or some other high energy compound, which is later depleted as the proton motive force runs down. The uncoupler effect also does not exclude coupling to other gradients, such as sodium or potassium.

As(III) efflux protein. In addition, CgAcr3-3 has a lower sequence similarity with other homologues. This may explain why CgAcr3-3 is not an active As(III) efflux protein. In addition, CgAcr3-3 has a lower sequence similarity with other Acr3 proteins (see alignments in supplemental Fig. S1), it is not regulated by the sequence similarity with other homologues (Lys72, Arg210, or Glu305; supplemental Fig. S1).

In A. metalliredigens, the gene for the Acr3 arsenite permease (AmAcr3) is adjacent to the genes for the ArsA-ATPase, and presumably they form an As(III) efflux pump complex, equivalent to the ArsAB system of E. coli (10, 32). However, Acr3 homologues lack a recognizable nucleotide binding domain, and therefore it is reasonable to assume that CgAcr3 proteins are secondary efflux carriers for As(III). How is CgAcr3-1 coupled to metabolic energy? The addition of the Δp uncoupler agent CCCP to C. glutamicum cells containing only the arsenite permease CgAcr3-1 prevented As(III) efflux (Fig. 3), suggesting that CgAcr3-1 is coupled to the proton motive force (Δp). However, it is still possible that CgAcr3-1 is coupled to chemical energy, either as ATP or some other high energy compound, which is later depleted as the proton motive force runs down. The uncoupler effect also does not exclude coupling to other gradients, such as sodium or potassium.

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Sb(III). Aquaglyceroporins are the nearly universal pathway for uptake of glycerol and other neutral molecules, including As(OH)₃ and Sb(OH)₃ (33). In *E. coli*, they are also responsible for As(III) uptake, where both GlpF and ArsB catalyze uptake and efflux, respectively, of Sb(III) at higher rates than As(III) (10). On the other hand, an Acr3 homologue from *S. oneidensis* appears to transport As(V) rather than As(III) (14). Therefore, the substrate specificity of Acr3s appears to differ among various homologues.

*C. glutamicum* might have a more limited substrate specificity than the yeast branch of the BART family. *C. glutamicum* is extremely sensitive to Sb(III) (20 μM) (34), but in this study, we observed that the level of Sb(III) uptake is 20-fold lower than As(III) in either intact cells or everted membrane (see above). On the other hand, an Acr3 homologue from *S. oneidensis* appears to transport As(V) rather than As(III) (14). Therefore, the substrate specificity of Acr3s appears to differ among various homologues.

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Even based on CgAcr3-1 topology (Fig. 2), little is known about the relation of individual residues to the function of Acr3 proteins. In a number of arsenic resistance proteins, including the ArsR repressor (27), the ArsD metallochaperone (35), and the ArsA ATPase (36), As(III) is bound by two or three cysteine residues. In these proteins, the As–S bonds are very strong, nearly covalent, and therefore the energy to release As(III) is very high. In contrast, ArsB arsenite permease has no conserved cysteines, and no thiolates are involved in As(III) translocation (29). Indeed, if As(III) were bound with high affinity to residues of a transport protein, the metalloid could not be released, and transport would not be possible. Thus, it is reasonable to predict that As(III) will be bound with relatively low affinity to residues within transmembrane segments in the case of CgAcr3-1. When the six very conserved hydrophilic residues in transmembrane segments of CgAcr3-1 (including a cysteine) were replaced with alanine, only the C129A and E305A substi-
tutions led to loss of transport activity. Although loss of function in a mutant does not clearly demonstrate that the indicated residue has a direct role in catalysis, it is a reasonable deduction to propose that the conserved cysteine and glutamic residues are involved in As(III) translocation. These amino acids are located in the middle of transmembrane segments (Fig. 2 and supplemental Fig. S1), and it is tempting to speculate that both residues serve as a selectivity filter for As(III). All other substitutions of Glu305 (each fulfilling different properties) also decreased As(III) uptake in everted membranes, suggesting that changes in charge or size of that residue alter the permease function, which affects As(III) translocation. It is also possible that Glu305 from CgAcr3-1 may be involved in H+ translocation. It is also possible that changes in charge or size of that residue alter the permease function, which has been proposed for other membrane proteins, hypotheses that can be examined in future studies.

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