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(大腸菌の L−アラニン排出輸送体 AlaE の機能解析)

Seryoung Kim

Doctoral dissertation

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Contents

I. Introduction

The cytoplasmic membrane of bacteria plays a crucial role(s) in maintaining intracellular homeostasis (1) that is balanced between solute-specific carrier- or transporter-mediated uptake and efflux of their substrates (1). In terms of amino acid metabolism, extensive biochemical and genetic studies have identified and characterized a large number of amino acid importers (2, 3, 4, 5, 6, 7, 8). In contrast, due in part to technical difficulties, studies on amino acid exporters lagged behind that of importers until the first identification of the lysine exporter, LysE, of *Corynebacterium glutamicum* (9). Since then, more than a dozen amino acid exporters and their homologues have been identified including threonine, isoleucine, and glutamic acid exporters in *C. glutamicum* (10, 11, 12); homoserine, cysteine, threonine, arginine, leucine, and aromatic amino acids exporters in *Escherichia coli* (13, 14, 15, 16, 17, 18, 19, 20, 21, 22); and the aspartate alanine exchanger in *Tetragenococcus halophilus* (23). Physiological roles of the amino acid exporters were assumed to ensure homeostatic maintenance of the intracellular amino acid pool by acting as a "safety valve" to avoid rapid increase of the intracellular amino acids to a toxic level and export of intercellular signal molecules such as a quorum sensing signal molecule, homoserine lactone (22, 24, 25, 26). However, a convincing experimental manifestation to explain these hypotheses remains elusive.

Recently, Hori in our laboratory identified the *alaE* (formerly *ygaW*) gene, which encodes the major L-alanine exporter in *E. coli* (27, 28). Striking features of AlaE are that the protein contains only 149 amino acid residues (Fig. 1-1), which is the smallest among the amino acid exporters identified to date, and that the *alaE* homologues were found to be conserved only in gamma- and alpha-proteobacteria (27, 28). Hori isolated the mutant lacking *alaE* and found that it was hypersusceptible to the L-alanine-containing dipeptide, Ala-Ala, suggesting that the mutant accumulated a

toxic level of intracellular L-alanine due to its L-alanine export defect (27, 29). Indeed, the *alaE*-deficient mutant derived from an L-Ala nonmetabolizing strain (MLA301) accumulated over 160 mM L-alanine in the cells in the presence of 6 mM Ala-Ala, whereas intracellular L-alanine in the *alaE*-overexpressing cell was below 40 mM under the same conditions (27, 28). In order to elucidate the physiological roles of the amino acid exporters including AlaE, it is essential to gain a profound understanding of the function of the exporters. For this purpose, I took the L-alanine exporter AlaE of *E. coli* and analyzed its transport activity in this study.

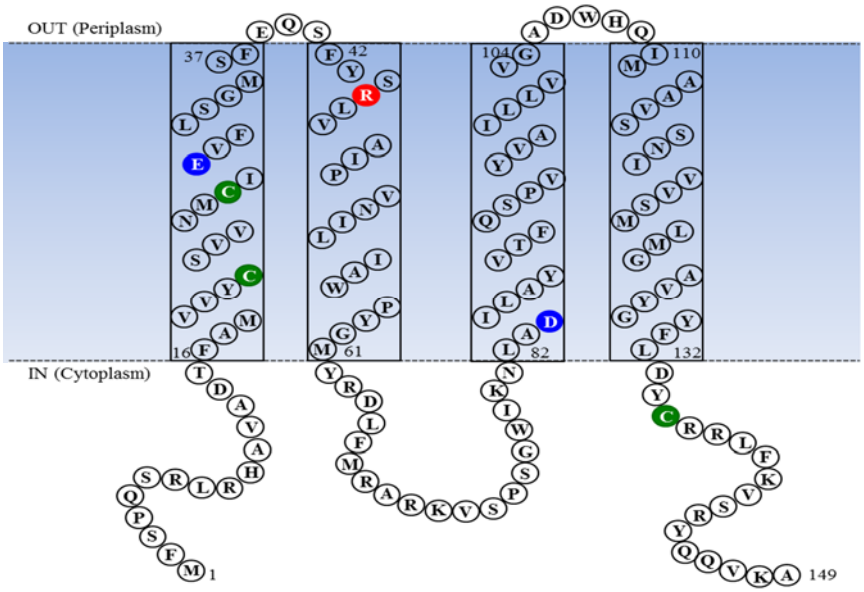


Figure 1-1. The secondary structure model of AlaE. AlaE is predicted to have four transmembrane segments by several algorithms used. This figure represents the membrane topology of AlaE predicted by the TMHMM program version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Transmembrane segments are represented by boxes. Cysteine residues and charged amino acid residues are highlighted. Amino acid residues are represented by one letter codes.

II. Evaluation of L-alanine export activity of AlaE in *in vivo* and *in vitro* assay systems

1. Introduction

In order to elucidate the physiological roles of the L-alanine exporter AlaE, it is essential to gain a detailed understanding of the function of AlaE. For this purpose, I analyzed the AlaE transport activity in *in vivo* and *in vitro* assay systems. As an *in vitro* system, I adopted a cell-free assay system using the inverted membrane vesicles prepared from *alaE*-overexpressing and *alaE*-deficient cells and determined radio-labeled L-alanine accumulation into the vesicles under the conditions of downhill, equilibrium, and uphill substrate concentration gradient.

2. Materials and methods

2-1) Bacterial strains, plasmids, and growth conditions

The *E. coli* strains and plasmids used in this study are listed in Table 2-1. Cells were grown aerobically at 37°C in L-broth containing 1% tryptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.2) or minimal medium (30) containing 22 mM glucose, 7.5 mM $(NH_4)_2SO_4$, 1.7 mM $MgSO_4$, 7 mM K_2SO_4, 22 mM NaCl and 100 mM sodium phosphate (pH 7.1). D-alanine (50 μg/ml), L-alanine (50 μg/ml), gentamicin (6.25 μg/ml), kanamycin (6.25 μg/ml), and chloramphenicol (12.5 μg/ml) were supplemented as needed. Growth was monitored by reading the turbidity at 660 nm (A_{660}).

2-2) L-alanine accumulation in intact cells

L-alanine accumulation in intact cells was determined as described previously with some modifications (8). Cells were grown in L-broth containing D-alanine (50 μg/ml) and appropriate antibiotics as described above at 37°C overnight, and harvested

by centrifugation at 8,900 x g for 10 min. Pellets were washed twice with minimal medium containing D-alanine (50 μg/ml), gentamicin (6.25 μg/ml), and kanamycin (6.25 μg/ml) and suspended in the same medium adjusting to the cell density to A660 0.5. After incubation at 37°C for two h, cells were collected by centrifugation as described above, suspended in the same ice-cold minimal medium adjusting the cell density to A_{660} 1.0, and kept on ice until use. A plastic tube containing 1.5 ml of cell suspension in the presence of 300 μg/ml of chloramphenicol was pre-incubated at 37°C for 10 min. The reaction was initiated by adding a 1.5-ml aliquot of the same minimal medium containing 20 μM L-[2,3-^{3}H]alanine (60 Ci/mmol, American Radiolabeled Chemical, USA) and terminated by filtering 100 μl aliquots through a membrane filter with a pore size of 0.22 μm, followed by washing twice with 3 ml each of pre-warmed medium. A membrane filter was placed in a plastic vial and immersed in 6 ml of the scintillant Filter-Count (PerkinElmer, USA). The radioactivity was counted after 24 h by the liquid scintillation counter, SLC-5001 (Hitachi Aloka Medical, Japan).

2-3) Preparation of inverted membrane vesicles

Inverted membrane vesicles were prepared as described previously (31). Briefly, cells grown in 150 ml of L-broth at 37°C overnight were diluted with a 1.5-L of fresh medium and the mixture was incubated on a Bio-shaker BR-300LF (TAITEC Co., Japan) at 100 reciprocal shaking per minute until the absorption at 660 nm reached 1.0. Cells were collected by centrifugation at 8,900 x g for 10 min at 4°C and washed twice with a solution containing 50 mM potassium phosphate and 5 mM $MgSO_4$ (pH 7.0). The cells suspended in a solution containing 50 mM potassium phosphate, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 20% glycerol (pH 7.0) were passed through a French pressure cell (AVESTIN, Canada) at 82.8 MPa. Unbroken cells and large cell debris were removed by centrifugation at 39,000 x g for 20 min at 4°C. Next, the supernatant

fraction was centrifuged (Beckman XL-90, USA) at 231,000 x g for 1.5 h at 4°C to collect the inverted membrane vesicles. Pellets were suspended in the same solution adjusting the protein concentration to 25 mg/ml, divided into small aliquots, quickly frozen with liquid N_2, and then kept at -80°C until use. For the preparation of L-alanine-loaded inverted membrane vesicles, cells were washed twice with a solution containing 50 mM potassium phosphate, 5 mM $MgSO_4$, and 200 mM L-alanine (pH 7.0) and then suspended in a solution containing 50 mM potassium phosphate, 5 mM $MgCl_2$, 1mM DTT, 20% glycerol, and 200 mM L-alanine (pH 7.0). The suspension was subjected to the French pressure cell treatment as above.

2-4) Uptake assay with inverted membrane vesicles

Uptake of [^{3}H]L-alanine into inverted membrane vesicles were carried out as follows. After incubation of 20 μl (0.5 mg protein equivalent) of the membrane vesicles for 2 min in a 25°C water bath, the reaction was started by adding 80 μl of a solution containing [^{3}H]L-alanine with or without 3.15 mM NADH (final concentration, 2.5 mM) in 50 mM potassium phosphate and 5 mM $MgSO_4$ (pH 7.0). An aliquot of the mixture was filtered through a 0.22 μm-pore size membrane filter (Millipore Co., USA) at the indicated time intervals, and washed twice with 3 ml of the pre-warmed (25°C) same buffer. Next, the membrane filters were immersed in 6 ml of the scintillant Filter-Count (PerkinElmer, USA) and the radioactivity was counted after 24 h by the liquid scintillation counter SLC-5001 (Hitachi Aloka Medical, Japan).

2-5) Effect of energy inhibitors on the active transport of L-alanine by inverted membrane vesicles

After incubation of 200 mM L-alanine-loaded inverted membrane vesicles (0.5 mg protein/20 μl) for 1 min 40 sec in a 25°C water bath, 2 μl of 125 mM NADH was

added and incubate for further 20 sec at 25°C. Then the reaction was started by adding a solution of 50 mM potassium phosphate (pH 7.0) and 5 mM $MgSO_4$ containing carbonyl cyamide *m*-chlorophenylhydrazone (CCCP) or dicyclohexylcarbodiimide (DCCD) to a final concentration of 20 μM or 50 μM, respectively, and [^{3}H]L-alanine (38 Ci/mmol, Moravek Biochemical, USA; final concentration, 0.34 μM). A final concentration of ethanol, in which CCCP and DCCD were dissolved as a stock solution, in the reaction mixture (100 μl) was below 0.5%. After 5 min of incubation at 25°C, an aliquot of the reaction mixture was filtered through a 0.22 μm-pore size membrane filter (Millipore Co., USA) and washed twice with 3 ml of the same pre-warmed buffer (50 mM potassium phosphate (pH 7.0) and 5 mM $MgSO_4$). Next, the membrane filters were immersed in 6 ml of the scintillant Filter-Count (PerkinElmer, USA) and the radioactivity was counted after 24 h by the liquid scintillation counter SLC-5001 (Hitachi Aloka Medical, Japan). Net AlaE-dependent [^{3}H]L-alanine accumulation in the vesicles was obtained by subtracting the accumulation in the vesicles prepared from *alaE*-deficient cells from that obtained from *alaE*-overexpressing cells.

2-6) Effects of D-alanine on the AlaE-mediated active transport of L-alanine

After incubation of 20 μl (0.5 mg protein equivalent) of the 200 mM L-alanine-loaded membrane vesicles for 2 min in a 25°C water bath, the reaction was started by adding 80 μl of a solution containing [^{3}H]L-alanine (final concentration, 0.34 μM), NADH (final concentration, 2.5 mM), and D-alanine in 50 mM potassium phosphate and 5 mM $MgSO_4$ (pH 7.0). The range of D-alanine concentration was set at 100 μM to 2.5 mM. The reaction mixture was incubated at 25°C for 5 min and the reaction was terminated by filtering an aliquot of the mixture (100 μl) through a 0.22 μm-pore size membrane filter (Millipore Co., USA) followed by washing twice with 3 ml of the pre-warmed buffer. Next, the membrane filters were immersed in 6 ml of the scintillant

Filter-Count (PerkinElmer, USA) and the radioactivity was counted after 24 h by the liquid scintillation counter SLC-5001 (Hitachi Aloka Medical, Japan). AlaE-dependent [^{3}H]L-alanine accumulation in the vesicles was obtained as described in the previous section and the value in the absence of a competitor was set to 100%.

2-7) Other techniques

Protein concentrations were determined by the method of Lowry et al. (32). SDS-PAGE was carried out by the method of Laemmli (33). Nucleotide sequences of the mutagenized *alaE* genes were determined by the dideoxy chain termination method (34).

3. Results

3-1) Outward transport of L-alanine in intact cells

As the *alaE* gene was identified to encode the L-alanine exporter (27, 28), I first determined time course accumulation of [^{3}H]L-alanine in intact cells producing wild-type AlaE (MLA301), cells lacking the *alaE* (MLA301Δ*alaE*) gene, and MLA301Δ*alaE*/pAlaE producing plasmid-borne AlaE (Fig. 2-1). The MLA301Δ*alaE* cells accumulated L-alanine in a time-dependent manner reaching 4.19 nmol/mg dry weight at 5 min. This might have been due to the functioning of active L-alanine importers, but non-functioning of the exporter. The MLA301 cells also accumulated L-alanine linearly reaching 2.09 nmol/mg dry weight at 5 min, which was approximately one-half that of MLA301Δ*alaE* cells. The *alaE*-deficient mutant harboring pAlaE accumulated about 55% L-alanine of the level in MLA301 in 5 min and that was roughly equivalent to one-quarter of that in the *alaE*-deficient cells. Although the level of intracellular L-alanine is the sum of the uptake and efflux of L-alanine, the different level of L-alanine accumulation in these strains was interpreted to

reflect the presence and absence of the *alaE* gene. The results were consistent with our earlier observation that levels of intracellular L-alanine in the presence of 6 mM Ala-Ala in MLA301Δ*alaE* and MLA301Δ*alaE*/pAlaE were higher and lower than that in MLA301 cells, respectively (27, 28).

3-2) L-alanine accumulation in the inverted membrane vesicles under a downhill solute gradient

To demonstrate L-alanine export directly in the cell-free system, I determined [^{3}H]L-alanine accumulation in inverted membrane vesicles prepared from MLA301Δ*alaE* and MLA301Δ*alaE*/pAlaE under a downhill solute gradient. Experiments were carried out to test four parameters that might affect the result: (i) time course of L-alanine accumulation; (ii) effect of the extravesicular L-alanine concentration; (iii) effect of the energy source that drives the efflux of L-alanine, and (iv) the impact of the presence or absence of the AlaE exporter. The time course of L-alanine accumulation was first determined in the inverted membrane vesicles prepared from cells lacking AlaE in the presence of an energy source and 200 mM extravesicular L-alanine. As shown in Fig. 2-2C, the cells accumulated only to the basal level of L-alanine. Similar experiments carried out using membrane vesicles prepared from cells with intact AlaE accumulated 27.1 nmol/mg protein in 5 min and the level was maintained for at least 10 min in the presence of 200 mM extravesicular L-alanine (Fig. 2-2A). This intravesicular accumulation of L-alanine appears to have been mediated by AlaE, since the vesicles prepared from MLA301Δ*alaE* accumulated negligible amounts of L-alanine under the same conditions (Fig. 2-2C). When the energy source to drive the active efflux was depleted from the assay system, the vesicles prepared from cells producing intact AlaE accumulated L-alanine to the level of 12.5 nmol/mg

protein in 5 min, which was less than half of the amount in the energized vesicles at 200 mM extravesicular L-alanine (Fig. 2-2B). This level of L-alanine accumulation may have been the result of downhill facilitated diffusion of the solute through the L-alanine carriers even without an energy source. Thus, it is clear that the vesicles prepared from cells with intact AlaE accumulated L-alanine in an energy-dependent manner. As the substrate concentration was varied from 50 mM to 200 mM, the vesicles from the AlaE-producing cells accumulated L-alanine increasingly in response to the extravesicular L-alanine concentration as expected (Fig. 2-2A).

3-3) L-alanine accumulation in the inverted membrane vesicles under an equilibrium solute state

Since the above-described experiments were carried out under conditions where the AlaE exporter catalyzed a downhill movement of L-alanine according to the substrate chemical potential, I next tested whether AlaE catalyzes L-alanine movement under conditions in which the internal and external solute concentrations were in equilibrium. For this purpose, I prepared the inverted membrane vesicles in the presence of 200 mM non-radioactive L-alanine. Then, I determined the [^{3}H]L-alanine accumulation in the vesicles in the presence of 200 mM extravesicular radiolabeled L-alanine. The L-alanine-loaded inverted membrane vesicles prepared from MLA301Δ*alaE*/pAlaE showed a clear energy-dependent accumulation of [^{3}H]L-alanine, reaching a plateau at 23.5 nmol/mg protein in 1 min (Fig. 2-3A). In contrast, the vesicles prepared from MLA301Δ*alaE* accumulated only 13.3 nmol/mg protein in 1 min followed by a slow leakage in the presence of NADH (Fig. 2-3B). Since this transient L-alanine accumulation likely occurred in an AlaE-independent manner, these values were subtracted from that of AlaE-mediated L-alanine accumulation. The values shown by a dotted line in Fig. 2-3B were likely attributable to genuine AlaE-

mediated transport activity. It is of interest to note that transient increase in the intravesicular L-alanine was similarly observed in the vesicles prepared from MLA301Δ*alaE*/pAlaE in the absence of NADH (Fig. 2-3A).

3-4) L-alanine accumulation in the inverted membrane vesicles under an uphill solute gradient

Next, I determined the [^{3}H]L-alanine accumulation in the 200 mM L-alanine-loaded membrane vesicles in the presence of only 0.34 μM extravesicular [^{3}H]L-alanine, a setup for an outward L-alanine chemical potential. The membrane vesicles prepared from MLA301Δ*alaE*/pAlaE rapidly accumulated [^{3}H]L-alanine reaching 43.3 fmol/mg protein within 1 min in the presence of NADH, and the level of labeled L-alanine was steadily maintained for at least the following 10 min (Fig. 2-4A). In contrast, only a trace amount of L-alanine accumulation was observed in the absence of the energy source (Fig. 2-4A). The inverted membrane vesicles prepared from MLA301Δ*alaE* showed a transient increment of intravesicular L-alanine, reaching 28 fmol/mg protein in 1 min in the presence of NADH; the level then declined to the basal level in 10 min (Fig. 2-4B). This energy-dependent transient L-alanine accumulation is likely due to exporters other than AlaE, such as YtfF, YddG, and YeaS as reported earlier (27, 28). Subtraction of this background of L-alanine accumulation in MLA301Δ*alaE* vesicles from that of MLA301Δ*alaE*/pAlaE showed the genuine AlaE-catalyzed energy-dependent uphill movement of L-alanine (Fig. 2-4B, dotted line). Taken together, these results unequivocally demonstrated that AlaE catalyzes the active export of L-alanine in an energy-dependent manner.

3-5) Effect of carbonyl cyamide *m*-chlorophenylhydrazone (CCCP) on the active transport of L-alanine by inverted membrane vesicles

To evaluate the nature of the energy that drives the active export of L-alanine by AlaE, I determined the [^{3}H]L-alanine accumulation in the 200 mM L-alanine-loaded membrane vesicles in the presence or absence of 20 μM of carbonyl cyamide *m*-chlorophenylhydrazone (CCCP) under an uphill solute gradient conditions. As shown in figure 2-5, accumulation of [^{3}H]L-alanine in the L-alanine-loaded vesicles prepared from MLA301Δ*alaE*/pAlaE decreased by about 74% in the presence of CCCP during the incubation time of 5 min as compared to that obtained in the absence of CCCP. In contrast, when 50 μM of N, N'-dicyclohexylcarbodiimide (DCCD), which inhibits the ATPase activity of *E. coli* membrane (35, 36, 37), was present in the assay system, almost the same level of [^{3}H]L-alanine was accumulated in the inverted membrane vesicles as observed for the untreated membrane vesicles (Fig. 2-5), indicating that an energy liberated from ATP hydrolysis is not a source of driving force for L-alanine export by AlaE. Taken together, these results strongly suggest that AlaE catalyzes active export of L-alanine using proton electrochemical potential.

3-6) Effect of D-alanine on the AlaE-mediated active transport of L-alanine

The above-described results demonstrated that AlaE catalyzes active export of L-alanine using proton motive force. To understand a physiological role of AlaE more profoundly, substrate specificity of this exporter needs to be clarified. I addressed this issue, as a first step, by asking of whether D-alanine could compete with [^{3}H]L-alanine accumulation in the L-alanine-loaded membrane vesicles in the presence of energy. As shown in figure 2-6, D-alanine inhibited the L-alanine accumulation in the inverted membrane vesicles in a dose-dependent manner, showing that D-alanine at a concentration of 0.1, 0.5, 1.0, and 2.5 mM inhibited [^{3}H]L-alanine accumulation in the

vesicles to a level of 59, 41, 26, and 18%, respectively, of that obtained in the absence of D-alanine. This indicated that 100 μM D-alanine (about 300-fold excess of labeled L-alanine) partially inhibited [^{3}H]L-alanine accumulation, but large amount of D-alanine (≥1.0 mM, more than about 3,000-fold excess) strongly inhibited the AlaE transport activity.

4. Discussion

In this study, I performed biochemical analyses to show L-alanine transport activity of AlaE by employing inverted membrane vesicles prepared from *alaE*-overexressing and *alaE*-deficient cells. The inverted membrane vesicles prepared from cells lacking AlaE transiently accumulated [^{3}H]L-alanine in the presence of NADH at one min followed by a slow decline (Figs. 2-3B and 2-4B). This initial accumulation was likely mediated by other L-alanine exporters, such as YtfF, YddG, and YeaS (27, 28). Decrease in intravesicular [^{3}H]L-alanine after the initial uptake in the membrane vesicles lacking AlaE might have been due to the L-alanine importers, CycA and LIV (8, 38, 39, 40). In contrast, a high level of intravesicular L-alanine was maintained in the L-alanine-loaded inverted membrane vesicles prepared from MLA301Δ*alaE*/pAlaE in the presence of NADH (Figs. 2-3A and 2-4A), indicating that AlaE exporter activity exceeded importer(s) activity under these conditions. These results clearly demonstrate that AlaE catalyzed the active export of L-alanine.

The inverted membrane vesicles prepared from MLA301Δ*alaE*/pAlaE transiently accumulated L-alanine in the L-alanine-loaded vesicles in the absence of NADH (Fig. 2-3A), but not in the vesicles prepared from cells lacking AlaE under an equilibrium solute potential (Fig. 2-3B). Therefore, this transient accumulation of L-alanine was likely mediated by AlaE (Fig. 2-3A). It is well known that transporters such as the lactose and melibiose permeases catalyze substrate exchange across the

membrane in the absence of energy (41, 42, 43, 44). In an analogy with this, I assume that this small increment of intravesicular L-alanine could have been a result of the L-alanine exchange reaction under the conditions tested (Fig. 2-3A).

Active transporters of bacteria are classified into two groups depending on their energy source: primary and secondary active transporters, which usually utilize a primary energy (chemical energy or light) or secondary energy (a free energy present in specific ion gradient or co-transported substrate gradients), respectively (45, 46). Several amino acid exporters, YeaS (16), ThrE (12), and RhtA (17), have been shown to be driven by proton motive force as assessed by amino acid excretion out of the intact cells in the absence or presence of CCCP. In contrast, CydDC of *E. coli* has been shown to export cysteine at the expense of ATP (20). In this study, I showed that AlaE utilizes proton electrochemical potential to export L-alanine (Fig. 2-5). It is noted that AlaE is the first case of ion-coupled amino acid exporters, to the best of my knowledge, showing that the transporters catalyze active export of their substrate in a cell-free system.

Furthermore about 300-fold excess of unlabeled D-alanine appeared to inhibit [^{3}H]L-alanine accumulation by about 41%. The results suggested that AlaE could have an ability to export D-alanine analog to some extent, a phenomenon similarly reported for other amino acid exporters (9, 47). However, whether D-alanine is actually been exported by AlaE in intact cells awaits further biochemical studies in the near future.

Table 2-1. List of strains and plasmids used

Strain or plasmid	Relevant property	Source or reference
Escherichia coli		
JM109	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17(r_k^- m_k^+), *e*14$^-$ (*mcrA*$^-$), *supE*44, *relA*1, Δ(*lac-proAB*)/F'[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	Laboratory strain
MLA301	MG1655 *alr*::*FRT*, *dadX*::*FRT*, *yfdZ*::*FRT*, *avtA*::GM, yfbQ::KM	(27, 29)
MLA301Δ*alaE*	MLA301 derivative with a deletion in the *alaE* gene	(27, 28)
Plasmid		
pSTV29	CP^r, *lacZ*, p15A ori	Takara
pAlaE	pSTV29 harboring a 1.0-kb PCR fragment of the *alaE* gene	(27, 28)

Abbreviations: GM, gentamicin; KM, kanamycin; CP, chloramphenicol

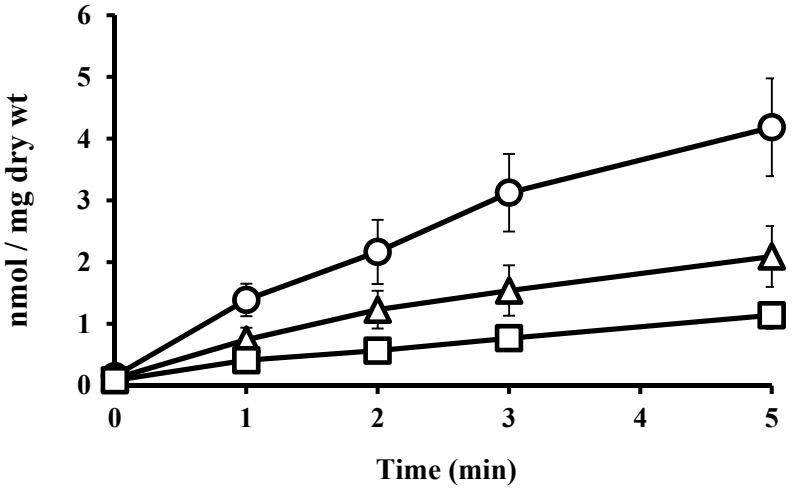


Figure 2-1. Accumulation of [^{3}H]L-alanine in intact cells. Cells were grown in minimal medium and suspended in the same medium. Transport assay was initiated by adding [^{3}H]L-alanine (60 Ci/mmol) to the final concentration of 10 μM and then incubating at 37°C. An aliquot (100 μl) of the cells suspension was filtered through a membrane filter with a 0.22 μm-pore size, and then washed twice with 3 ml of pre-warmed minimal medium. Values presented are the means of three independent experiments with standard deviations. Symbols: **▲,** MLA301; **○,** MLA301Δ*alaE*; **□,** MLA301Δ*alaE*/pAlaE.

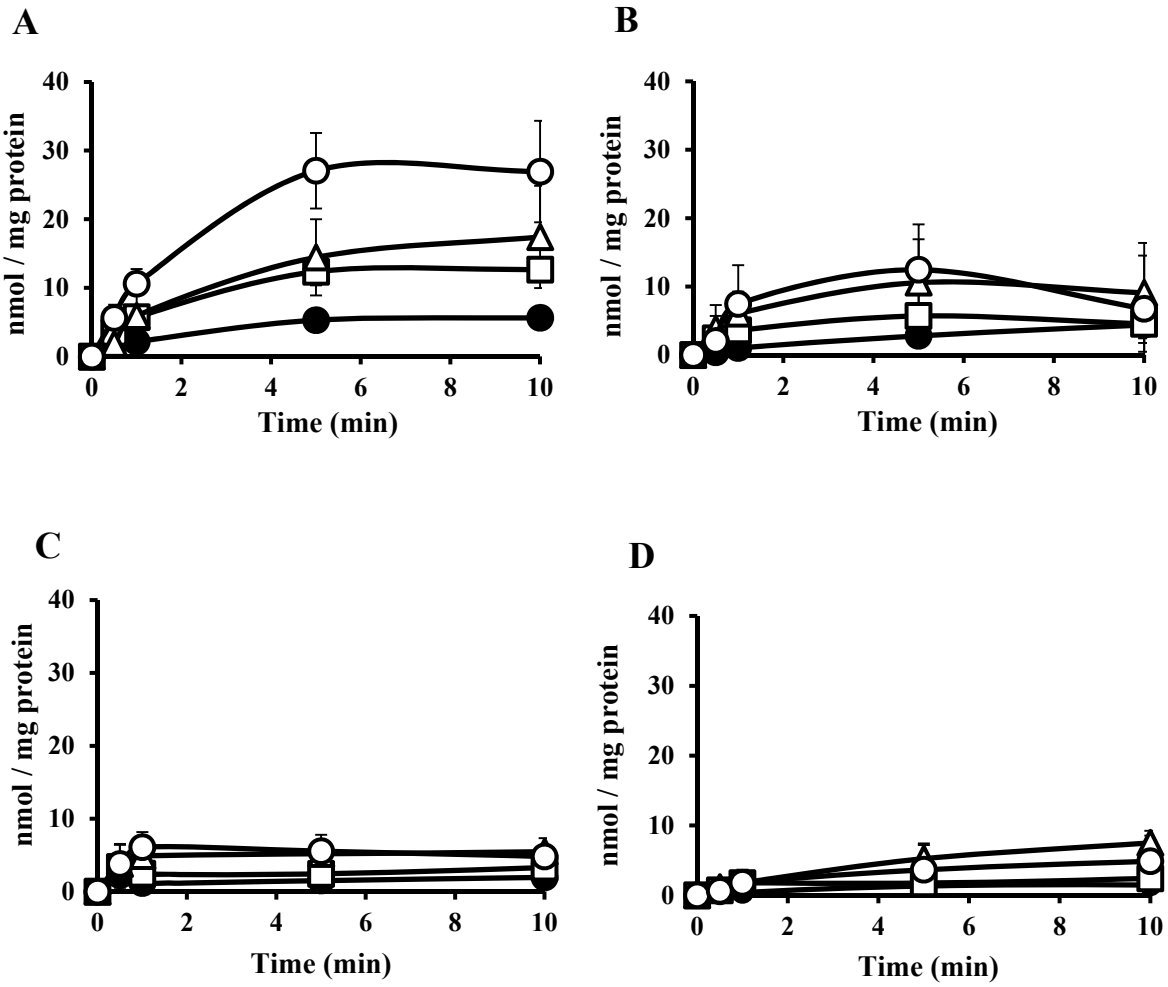


Figure 2-2. Accumulation of [^{3}H]L-alanine (60 Ci/mmol) in the inverted membrane vesicles under a downhill solute potential. The inverted membrane vesicles were prepared from MLA301Δ*alaE*/pAlaE (A and B) and MLA301Δ*alaE* (C and D). [^{3}H]L-alanine accumulation was assayed in the presence (A and C) and absence (B and D) of 2.5 mM NADH. Extravesicular L-alanine concentrations; ●, 50 mM; □, 100 mM; ∆,150 mM; and ○, 200 mM. Values presented are the means of more than three independent experiments with standard deviations.

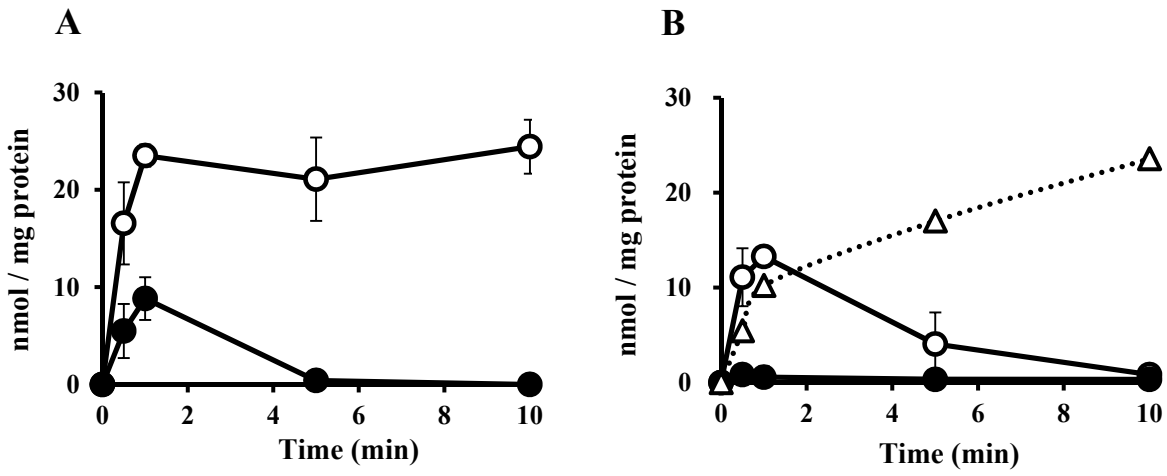


Figure 2-3. Accumulation of [^{3}H]L-alanine (60 Ci/mmol) in the L-alanine-loaded inverted membrane vesicles under an equilibrium solute potential. L-alanine-loaded inverted membrane vesicles were prepared from MLA301Δ*alaE*/pAlaE (A) and MLA301Δ*alaE* (B). Extravesicular L-alanine concentration was set to be equivalent to that of intravesicular L-alanine (200 mM). Symbols: ○, 2.5 mM NADH; ●, without NADH. Dotted line in (B) shows AlaE-mediated L-alanine accumulation obtained by subtracting the values in MLA301Δ*alaE* from those in MLA301Δ*alaE*/pAlaE in the presence of NADH. Values presented are means of three independent experiments with standard deviations.

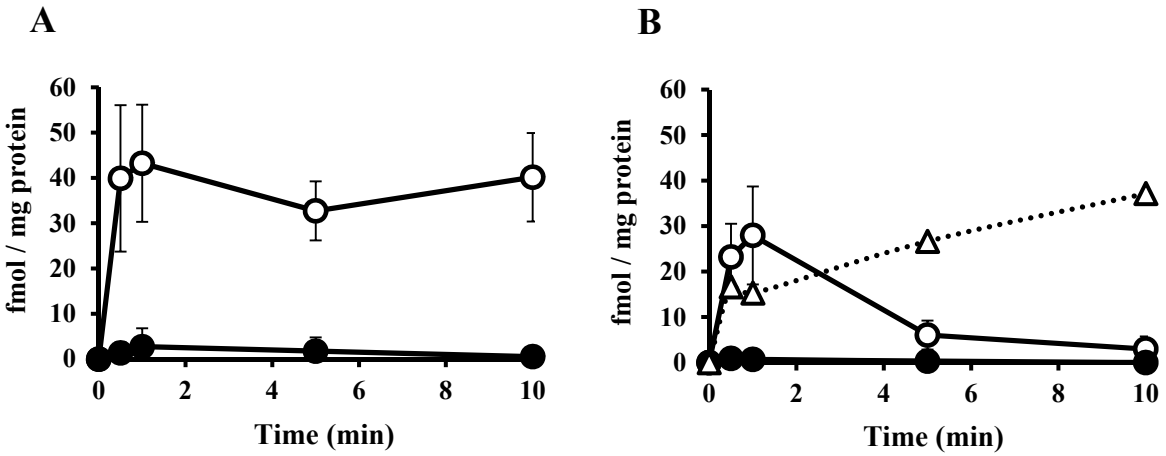
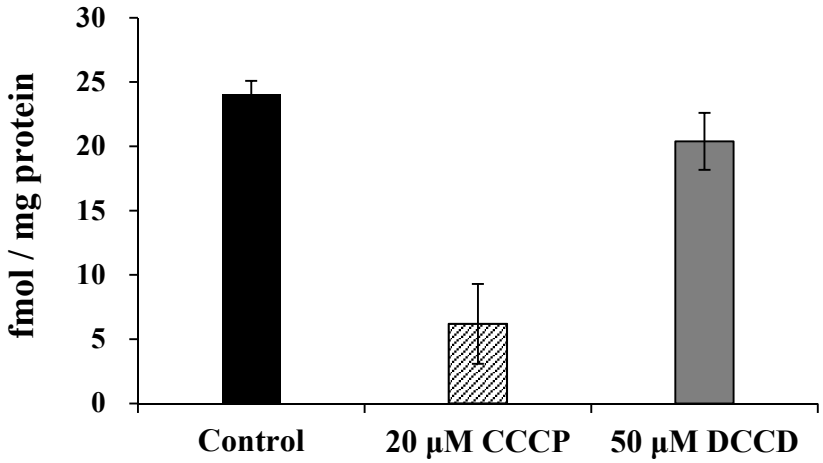


Figure 2-4. Accumulation of [^{3}H]L-alanine (60 Ci/mmol) in the L-alanine-loaded inverted membrane vesicles under an uphill solute potential. Inverted membrane vesicles prepared from MLA301Δ*alaE*/pAlaE (A) and MLA301Δ*alaE* (B) were loaded with 200 mM L-alanine, and the extravesicular L-alanine concentration was set to 0.34 μM with radioactive L-alanine. Symbols: ○, 2.5 mM NADH; ●, without NADH. Dotted line in (B) shows AlaE-mediated L-alanine accumulation obtained by subtracting the values in MLA301Δ*alaE* from those in MLA301Δ*alaE*/pAlaE in the presence of NADH. Values presented are the means of three independent experiments with standard deviations.



Figrue 2-5. Effect of energy inhibitors on the accumulation of [^{3}H]L-alanine in the L-alanine-loaded inverted membrane vesicles under an uphill solute potential. [^{3}H]L-alanine accumulation was determined after 5 min of incubation as described in Materials and methods. The values represent AlaE-mediated [^{3}H]L-alanine (38 Ci/mmol) accumulation by subtracting values obtained with MLA301Δ*alaE* vesicles from those in MLA301Δ*alaE*/pAlaE in the presence of NADH. Black box, control; hatched box, 20 μM CCCP; grey box, 50 μM DCCD. Values presented are the means of three independent experiments with standard deviations.

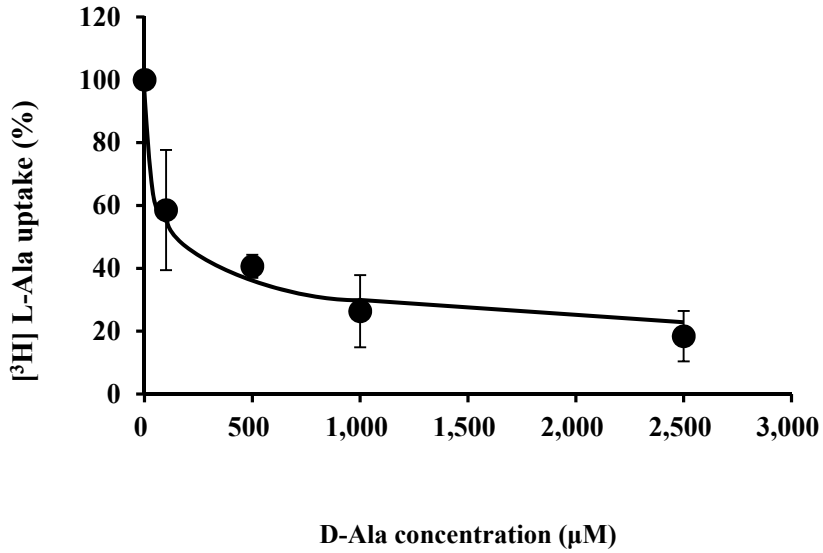


Figure 2-6. Effect of unlabeled D-alanine on the AlaE-mediated active transport of L-alanine. Accumulation of [^{3}H]L-alanine (38 Ci/mmol) in 200 mM L-alanine-loaded membrane vesicles was determined as described in Materials and methods in the presence of various amount of unlabeled D-alanine (0.1 mM to 2.5 mM). [^{3}H]L-alanine accumulation was calculated by subtracting the values obtained with MLA301Δ*alaE* vesicles from those in MLA301Δ*alaE*/pAlaE in the presence of NADH. The value obtained in the absence of D-alanine was set to 100%.

III. Construction and evaluation of cysteine-less AlaE

1. Introduction

For further understanding of the molecular mechanisms of AlaE-mediated L-alanine export, I employed the cysteine-scanning approach, a well-established technique to decipher the function and topology of membrane proteins (48). The very first step towards such studies would be to confirm that the cysteine-less derivative of a target protein of interest retains its transport activity. AlaE contains three cysteine residues, two of which, residues 22 and 28, are predicted to be located in the first transmembrane segment, and residue 135 is in the cytoplasmic carboxyl terminal region (Fig 1-1) (27, 28). To see the role of cysteine residues in the function of AlaE, we constructed three individual AlaE mutants replacing a single cysteine residue with alanine, and one mutant having triple cysteine-to-alanine mutations. Subsequently, I assessed their L-alanine export activity by determining MIC of Ala-Ala for *alaE*-deficient cells expressing each individual AlaE variant. In addition, I evaluated the substrate transport activity of the cysteine-less AlaE using inverted membrane vesicles prepared from *alaE*-deficient cells expressing the AlaE variant.

2. Materials and methods

2-1) Bacterial strains, plasmids, and growth conditions

The *E. coli* strains and plasmids used in this study are listed in Table 3-1. Cells were grown aerobically at 37°C in L-broth containing 1% tryptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.2) or minimal medium (30) containing 22 mM glucose, 7.5 mM $(NH_4)_2SO_4$, 1.7 mM $MgSO_4$, 7 mM K_2SO_4, 22 mM NaCl and 100 mM sodium phosphate (pH 7.1). D-alanine (50 μg/ml), L-alanine (50 μg/ml), gentamicin (6.25

μg/ml), kanamycin (6.25 μg/ml), and chloramphenicol (12.5 μg/ml) were supplemented as needed. Growth was monitored by reading the turbidity at 660 nm (A_{660}).

2-2) Site-directed mutagenesis and construction of the cysteine-less *alaE* gene

Cysteine residues of AlaE were replaced with alanine or serine by site-directed mutagenesis using primer pairs encoding a mismatch codon for alanine as shown in Table 3-2. Cysteine-less *alaE* gene was constructed serially by using primer sets, dC22A-F/C22A-R, C28A-F/C28A-R, and C135A-F/C135A-R, yielding pC22A-28A-135A. At each mutagenesis step, the PCR products were amplified by PrimeSTAR (Takara, Japan), digested with *Dpn*I and transformed into *E. coli* JM109. Plasmids were isolated from transformants grown on L-agar medium containing chloramphenicol (12.5 μg/ml) and the entire *alaE* gene was sequenced to ensure that no secondary mutation was present.

2-3) Determination of the minimum inhibitory concentration (MIC) of L-alanyl-L-alanine (Ala-Ala)

The minimum inhibitory concentration of Ala-Ala was determined as a measure of L-alanine transport activity. MLA301Δ*alaE* cells harboring the wild-type or cysteine-less AlaE-bearing plasmid were grown in L-broth containing 50 μg/ml D-alanine, 6.25 μg/ml gentamicin, 6.25 μg/ml kanamycin, and 12.5 μg/ml chloramphenicol at 37°C overnight. Then the cells were harvested, washed twice with 0.85% NaCl, and suspended in the same solution. Approximately 1~3 x 10^4 cells per 5 μl were spotted using a Microplanter MIT-P (Sakuma Co., Japan) onto minimal agar medium containing a two-fold serially diluted Ala-Ala. Plates were incubated at 37°C and MICs were scored after 24 and 44 h of incubation.

2-4) Preparation of inverted membrane vesicles

Inverted membrane vesicles were prepared as described previously (31). Briefly, cells grown in 150 ml of L-broth at 37°C overnight were diluted with a 1.35-L of fresh medium and the mixture was incubated on a Bio-shaker BR-300LF (TAITEC Co., Japan) at 100 reciprocal shaking per minute until the absorption at 660 nm reached 1.0. Cells were collected by centrifugation at 8,900 x g for 10 min at 4°C and washed twice with a solution containing 50 mM potassium phosphate and 5 mM $MgSO_4$ (pH 7.0). The cells suspended in a solution containing 50 mM potassium phosphate, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 20% glycerol (pH 7.0) were passed through a French pressure cell (AVESTIN, Canada) at 82.8 MPa. Unbroken cells and large cell debris were removed by centrifugation at 39,000 x g for 20 min at 4°C. Next, the supernatant fraction was centrifuged (Beckman XL-90, USA) at 231,000 x g for 1.5 h at 4°C to collect the inverted membrane vesicles. Pellets were suspended in the same solution adjusting the protein concentration to 25 mg/ml, divided into small aliquots, quickly frozen with liquid N_2, and then kept at -80°C until use. For the preparation of L-alanine-loaded inverted vesicles, cells were washed twice with a solution containing 50 mM potassium phosphate, 5 mM $MgSO_4$, and 200 mM L-alanine (pH 7.0) and then suspended in a solution containing 50 mM potassium phosphate, 5 mM $MgCl_2$, 1mM DTT, 20% glycerol, and 200 mM L-alanine (pH 7.0). The suspension was subjected to cell disruption with the French pressure cell as described above.

2-5) Uptake assay with inverted membrane vesicles

Uptake of [^{3}H]L-alanine into inverted membrane vesicles were carried out as follows. After incubation of 20 µl (0.5 mg protein equivalent) of the membrane vesicles for 2 min in a 25°C water bath, the reaction was started by adding 80 µl of a solution containing [^{3}H]L-alanine (60 Ci/mmol, American Radiolabeled Chemical, USA)

with or without 3.15 mM NADH (final concentration, 2.5 mM) in 50 mM potassium phosphate and 5 mM $MgSO_4$ (pH 7.0). An aliquot of the mixture (100 μl) was filtered through a 0.22 μm-pore size membrane filter (Millipore Co., USA) at the indicated time intervals, and washed twice with 3 ml of the same buffer. Next, the membrane filters were immersed in 6 ml of the scintillant Filter-count (PerkinElmer, USA) and the radioactivity was counted after 24 h by the liquid scintillation counter SLC-5001 (Hitachi Aloka Medical, Japan)

2-6) Construction of histidine-tagged *alaE* genes

A hexahistidine tag was added to the C-terminal end of AlaE using primer set His6-F/His6-R (Table 3-2) and each *alaE*-bearing plasmid as the template by the In-Fusion cloning system (Takara, Japan) according to the manufacturer's instructions. The resulting plasmids isolated from the transformants were sequenced to verify the presence of hexahistidine codons and absence of undesired mutations.

2-7) Preparation of the membrane fraction and immunoblotting analysis

Membrane fractions were isolated as described previously (27, 28). Briefly, transformants harboring the wild-type or the cysteine-less AlaE-encoding plasmid were grown in L-broth containing 50 μg/ml D-alanine, 6.25 μg/ml gentamicin, 6.25 μg/ml kanamycin, and 12.5 μg/ml chloramphenicol. Cells were harvested, washed twice with 50 mM Tris-HCl (pH 8.0), and suspended in the same buffer containing 1 mM phenylmethanesulfonyl fluoride (Wako Pure Chemical Industry, Japan). The suspension was subjected to sonic oscillation by 15-sec exposure and 45-sec intermittent cooling in an ice-bath at the maximum output using a Bioruptor (model UCD-200T, CosmoBio Co., Japan). The oscillation was continued until the cell suspension became translucent. The sonic extracts were centrifuged at 15,000 x g for

20 min at 4°C and the supernatant fraction was centrifuged again at 100,000 x g for 1 h at 4°C. The resulting membrane pellets were suspended in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) (pH 7.4) (49), mixed with the electrophoresis sample buffer and heated in a boiling water bath for 5 min (33). The membrane fraction containing 1 μg of proteins was subjected to SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane (Immobion-PVDF, Millipore, USA) using a blotting apparatus (model WSE-4020, ATTO, Japan), and probed with mouse monoclonal antibody raised against hexahistidine (Wako Pure Chemical Industries, Japan).

3. Results

3-1) MIC of Ala-Ala in the cysteine-less AlaE as a measure of L-alanine export

The above-described results firmly demonstrated that AlaE of *E. coli* is an L-alanine exporter, which is likely to play a crucial role in amino acid metabolism and homeostasis. To gain insights into the molecular mechanism by which this small membrane protein exports L-alanine efficiently, molecular dissection of the carrier protein is essential. As an entry to such studies, I first tested the effect of the replacement of cysteine residues with alanine. The wild-type AlaE has three cysteine residues at the 22nd, 28th, and 135th positions (Fig. 1-1). All three cysteine residues were converted to alanine yielding the pC22A-28A-135A plasmid.

It was reported previously that the *alaE* deletion mutant renders the cells hypersusceptible to the dipeptide Ala-Ala implying that the peptide MIC determination can be a simple and sensitive method for monitoring L-alanine exporter activity in intact cells. Thus, I determined the MIC of Ala-Ala in MLA301Δ*alaE*/pC22A-28A-135A (Table 3-3) according to the method of Hori *et al.* (27, 28, 29). The MIC of Ala-Ala for MLA301Δ*alaE*/pC22A-28A-135A at 44 h of incubation appeared at a level over 10

mg/ml, which was indistinguishable from that in MLA301 and MLA301Δ*alaE*/pAlaE. The results indicated clearly that cysteine-less AlaE retained L-alanine-exporting activity.

The MICs of Ala-Ala in MLA301, MLA301Δ*alaE*, MLA301Δ*alaE*/pSTV29 and MLA301Δ*alaE*/pAlaE at 24 h of incubation appeared to be 156 μg/ml, <1.25 μg/ml, <1.25 μg/ml, and >10 mg/ml, respectively. The results were essentially consistent with the results obtained at 44 h of incubation. The MIC value in the cells bearing cysteine-less AlaE was half that of MLA301Δ*alaE*/pAlaE at 24 h, suggesting that the cysteine-less AlaE has a slightly lower activity than the wild-type AlaE.

3-2) L-alanine accumulation in the inverted membrane vesicles prepared from cysteine-less AlaE-expressing cells under a downhill solute gradient

I next evaluated L-alanine export activity of cysteine-less AlaE using the inverted membrane vesicles prepared from MLA301Δ*alaE*/pC22A-28A-135A. In the presence of NADH and 200 mM extravesicular L-alanine, the vesicles accumulated [^{3}H]L-alanine in a time-dependent manner and the intravesiclular L-alanine attained the level of 23.8 nmol/mg protein at 10 min (Fig. 3-1A). As the extravesicular L-alanine concentration was lowered to 100 and 50 mM, the levels of L-alanine accumulation at 10 min were 11.7 and 5.8 nmol/mg protein, respectively, indicating that the transport occurred in a substrate concentration-dependent manner (Fig. 3-1A). The transport activity of the mutant AlaE seemed to be slightly lower than that of the wild-type AlaE (Fig. 3-1A, compared to Fig. 2-2A). In the absence of the energy source, the L-alanine accumulated amounts in the vesicles were also slightly lower than that of wild-type AlaE (Fig. 3-1B, compared to Fig. 2-2B).

3-3) L-alanine accumulation in the inverted membrane vesicles prepared from the cysteine-less AlaE-expressing cells under an equilibrium state and an uphill solute gradient

The above-described results might have been obtained because the triple mutation (C22A C28A C135A) caused a subtle distortion of AlaE's tertiary structure and the protein simply functioned as the L-alanine channel, which facilitated the energy-independent diffusion of L-alanine. To clarify such ambiguity, L-alanine uptake experiments were carried out under both an equilibrium state and a downhill solute gradient.

The inverted membrane vesicles were loaded with 200 mM L-alanine and the extravesicular L-alanine were set to the equilibrium concentration of 200 mM. Accumulation of [^{3}H]L-alanine at 1 min in the absence of NADH was 21 nmol/mg protein and the level of intravesicular L-alanine decreased to 3.6 and <1 nmol/mg protein at 5 and 10 min (Fig. 3-2A), respectively. This transient accumulation of L-alanine in the absence of NADH was about twice the level obtained in the wild-type AlaE (Fig. 2-3A). In the presence of NADH, the vesicle accumulated 25.4, 30.6, and 23.6 nmol/mg protein at 1, 5, and 10 min, respectively (Fig. 3-2A). Since the values obtained in the presence of NADH were more or less close to those of similar experiments using the wild-type AlaE (Fig. 2-3A), I concluded that the triple AlaE mutant retained its L-alanine transport activity.

Next, the cysteine-less AlaE-bearing inverted membrane vesicles were subjected to the uphill L-alanine uptake experiment by loading the vesicles with 200 mM L-alanine and setting the extravesicular [^{3}H]L-alanine to the concentration of 0.34 μM. In the absence of NADH, a transient L-alanine uptake was observed in which the amount of L-alanine accumulation was 15.5 fmol/mg protein at 1 min, declining to the basal level at 5 and 10 min (Fig. 3-2B). In the presence of an energy source, the

vesicles accumulated to the level of over 30 fmol/mg protein within 1 min and this level of intravesicular L-alanine was maintained for at least 10 min, indicating again that the cysteine-less AlaE retained its function.

3-4) Impacts of cysteine-22 mutation on the AlaE function

Although cysteine-less AlaE appeared to possess L-alanine export activity, simultaneous replacement of three cysteine residues with alanine had slightly compromised impact on the AlaE functionality as described above (Table 3-3, Fig. 3-1). Hence, I asked a question of which cysteine-to-alanine mutation in the cysteine-less AlaE (C22A C28A C135A) is responsible for this slight functional change. To address this issue, I constructed three mutant *alaE* genes each having only a single cysteine-to-alanine mutation and determined MIC of Ala-Ala for MLA301Δ*alaE* expressing individual AlaE variant, C22A, C28A, or C135A. All single AlaE mutants showed MIC of >10 mg/ml at 44 h of incubation, indicating that these individual single mutants retained L-alanine transport activity. However, close examination of the data revealed that MIC of Ala-Ala in MLA301Δ*alaE*/pC22A was 39 μg/ml at 24 h of incubation, whereas that in MLA301Δ*alaE*/pC28A and MLA301Δ*alaE*/pC135A appeared to be 10 mg/ml and 2.5 mg/ml, respectively (Table 3-3). The results suggested that a cysteine-to-alanine mutation at position 22 has the most profound effect on the AlaE activity among these three residues. When this cysteine residue was replaced with serine (C22S), a residue with similar size and chemical properties to cysteine, instead of alanine, MLA301Δ*alaE*/pC22S showed a significantly higher MIC value (10 mg/ml), a level similar to that of wild-type AlaE, as compared to the AlaE variant C22A at 24 h of incubation (Table 3-3). I next tested a possibility whether the cysteine-to-serine mutation at position 22 (C22S) might provide an improvement in the slightly compromised activity of the cysteine-less AlaE (C22A C28A C135A) by replacing a

C22A with C22S in the triple cysteine-less AlaE. Unexpectedly, MLA301Δ*alaE* expressing the cysteine-less AlaE (C22S C28A C135A) showed almost the same MIC toward Ala-Ala as that of MLA301Δ*alaE*/pC22A-28A-135A, suggesting that cysteine 22 in the transmembrane segment is important for the transport activity among these three cysteine residues.

3-5) Expression of the cysteine-less AlaE tagged with hexahistidine

The vesicles bearing the cysteine-less AlaE exhibited slightly lower L-alanine transport activity than those containing wild-type AlaE and enhanced transient L-alanine accumulation compared with those bearing the wild-type AlaE (Figs. 2-2, 3-1, and 3-2). One may argue, therefore, that replacement of the cysteine residues with alanine affected the level of mutant *alaE* expression, that is a reduced expression level of the cysteine-less AlaE could result in a decrease in its activity. To clarify this ambiguity, I constructed AlaE with a hexahistidine residue attached at the carboxyl terminal end, and confirmed that the hexahistidine-tagged wild-type and various AlaE variants including cysteine-less AlaE possessed transport activity comparable with that of the respective hexahistidine-less AlaEs by determining the MIC of Ala-Ala (data not shown). Membrane fractions were prepared from MLA301Δ*alaE* bearing hexahistidine-tagged AlaEs and subjected to SDS-polyacrylamide gel electrophoresis. The hexahistidine-tagged AlaEs were probed by the Western blotting method using a mouse monoclonal antibody raised against hexahistidine (Fig. 3-3). The expression of cysteine-less AlaE as well as individual single cysteine-to-alanine variant AlaEs appeared to be comparable with that of the wild-type AlaE.

4. Discussion

To pursue the molecular mechanism of L-alanine export via AlaE, I constructed cysteine-less AlaE and the mutant AlaE was tested for L-alanine transport activity *in vivo* and *in vitro* assay systems. The MIC of Ala-Ala in cells producing cysteine-less AlaE was found to be a slightly lower value than that in the cells producing the wild-type AlaE (Table 3-3). This slightly compromised function of the cysteine-less AlaE was confirmed *in vitro* by the downhill L-alanine transport assay (Fig. 3-1), in which the levels of L-alanine accumulation in the vesicles prepared from cysteine-less AlaE cells was slightly lower than that of the wild-type AlaE (Figs. 2-2A and 3-1A). These results suggested that replacement of all the cysteine residues with alanine had a subtle impact on the structure and function of AlaE. It is of interest to note that the transient accumulation of L-alanine in the inverted membrane vesicles containing cysteine-less AlaE was enhanced about 2-fold compared with that of vesicles containing the wild-type AlaE in the absence of an energy source under equilibrium conditions (Figs. 2-3A and 3-2A). The results suggested that replacement of cysteine residues with alanine might have had some impact on an AlaE transport step, leading to the increased transient accumulation under conditions tested. However, the reason why the L-alanine level decreased to the basal level under equilibrium conditions remains obscure at present.

It is also of interest that, in the uphill transport assay system, transient L-alanine accumulation in the inverted membrane vesicles prepared from cysteine-less AlaE-producing cells was observed in the absence of an energy source (Fig. 3-2B). Since the outward concentration gradient of non-labeled L-alanine is present in the assay system, the transient uptake of labeled L-alanine into the L-alanine-loaded inverted membrane vesicles might have been the result of a counterflow, a transport mode similar to that observed with lactose permease (39, 42, 43). The result is in line with

the prediction that the cysteine-to-alanine mutation may have influenced the function of AlaE. Regarding this slight effect on the functionality of the cysteine-less AlaE, a single mutation at position 22 (C22A) exerted the strongest impact on the activity of AlaE among three individual single cysteine mutants, C22A, C28A and C135A, since MLA301Δ*alaE*/pC22A showed strikingly reduced MIC toward Ala-Ala at 24 h of incubation (Table 3-3). I speculated therefore that the mutation at position 22 could be responsible for the slightly compromised function of the cysteine-less AlaE and this cysteine-to-alanine mutation might affect a transport step mediated by AlaE. Further in-depth biochemical analyses of the AlaE variants at position 22 could provide clues for further understanding of the mechanism by which AlaE exports L-alanine.

Taken together, cysteine-less AlaE constructed in this chapter appeared to retain L-alanine transport activity. Furthermore, replacement of all cysteine residues present in AlaE with alanine might somehow affect the symmetrical AlaE transport function. Therefore, the cysteine-less AlaE variant is an excellent tool for further structural and functional analyses of AlaE.

Table 3-1. List of strains and plasmids used

Strain or plasmid	Relevant property	Source or reference
Escherichia coli		
JM109	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17(r_K^- m_K^+), *e*14$^-$(*mcrA*$^-$), *supE*44, *relA*1, Δ(*lac-proAB*)/F'[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	Laboratory strain
MLA301	MG1655 *alr*::*FRT*, *dadX*::*FRT*, *yfdZ*::*FRT*, *avtA*::GM, yfbQ::KM	(27, 29)
MLA301Δ*alaE*	MLA301 derivative with a deletion in the *alaE* gene	(27, 28)
Plasmid		
pSTV29	CP^r, *lacZ*, p15A ori	Takara
pAlaE	pSTV29 harboring a 1.0-kb PCR fragment of the *alaE* gene	(27, 28)
pC22A	pAlaE derivative with single Cys replacement at position 22 with Ala	This study
pC28A	pAlaE derivative with single Cys replacement at position 28 with Ala	This study
pC135A	pAlaE derivative with single Cys replacement at position 135 with Ala	This study
pC22S	pAlaE derivative with single Cys replacement at position 22 with Ser	This study
pC22S-28A-135A	pAlaE derivative with triple Cys replacement at position 22 with Ser , 28 and 135 with Ala	This study

pC22A-28A-135A	pAlaE derivative with triple Cys replacement at position 22, 28, 135 with Ala	This study
pAlaE-His	pAlaE derivative with a hexahistidine residue attached at the C-terminal end of the *alaE* gene	This study
pC22A-His	pC22A derivative possessing hexa histidine at the C-terminal of the *alaE* gene	This study
pC28A-His	pC28A derivative possessing hexa histidine at the C-terminal of the *alaE* gene	This study
pC135A-His	pC135A derivative possessing hexa histidine at the C-terminal of the *alaE* gene	This study
pC22S-His	pC22S derivative possessing hexa histidine at the C-terminal of the *alaE* gene	This study
pC22S-28A-135A-His	pC22S-28A-135A derivative with a hexahistidine residue attached at the C-terminal end of the *alaE* gene	This study
pC22A-28A-135A-His	pC22-28-135A derivative with a hexahistidine residue attached at the C-terminal end of the *alaE* gene	This study

Abbreviations: GM, gentamicin; KM, kanamycin; CP, chloramphenicol

Table 3-2. Primers used in this study

Primer	Nucleotide sequence	Codon change
C22A-F	5'-GTTGTTTACGCTTCTGTCGTGAACATGTG-3'	TGT→GCT
dC22A-F	5'-TTGTTTACGCTTCTGTCGTGAACATGGC-3'	TGT→GCT
C22A-R	5'-AGAAGCGTAAACAACCATCGCGAACG-3'	ACA→AGC
C22S-F	5'-TTGTTTACTCTTCTGTCGTGAACATG-3'	TGT→TCT
C22S-R	5'-AGAAGAGTAAACAACCATCGCGAACG-3'	ACA→AGA
C28A-F	5'-AACATGGCTATTGAAGTTTTCCTCTCCG-3'	TGT→GCT
C28A-R	5'-TTCAATAGCCATGTTCACGACAGAACAG-3'	ACA→AGC
C135A-F	5'-TTATGCCCGCCGACTGTTTAAAGTCAGCCG-3'	TGC→GCC
C135A-R	5'-AGTCGGCGGGCATAATCGAGGAAGTAGCC-3'	GCA→GGC
His6-F	5'-CACCACCATCACCATCACTGACTCTTCTTTTC GCGACTGG-3'	-
His6-R	5'-GTGATGGTGATGGTGGTGGGCTTTTACCTGC TGGTAACGG-3'	-

The codon change is shown by the underlined font. dC22A-F is a primer for construction of a double mutant (C22A C28A) where a plasmid pC28A is used as the template. Underlined sequences of His6-F and His6-R represent hexahistidine codons.

Table 3-3. MIC of L-alanyl-L-alanine in the strain used

Strain	MIC (μg/ml)	
	24 h	44 h
MLA301	156	>10,000
MLA301Δ*alaE*	<1.25	2.5
MLA301Δ*alaE*/pSTV29	<1.25	<1.25
MLA301Δ*alaE*/pAlaE	> 10,000	>10,000
MLA301Δ*alaE*/pC22A	39	>10,000
MLA301Δ*alaE*/pC28A	10,000	>10,000
MLA301Δ*alaE*/pC135A	2,500	>10,000
MLA301Δ*alaE*/pC22S	10,000	>10,000
MLA301Δ*alaE*/pC22A-28A-135A	5,000	>10,000
MLA301Δ*alaE*/pC22S-28A-135A	5,000	>10,000

The MIC was scored after 24 h and 44 h of incubation. The MIC values presented are the results of more than three independent assays.

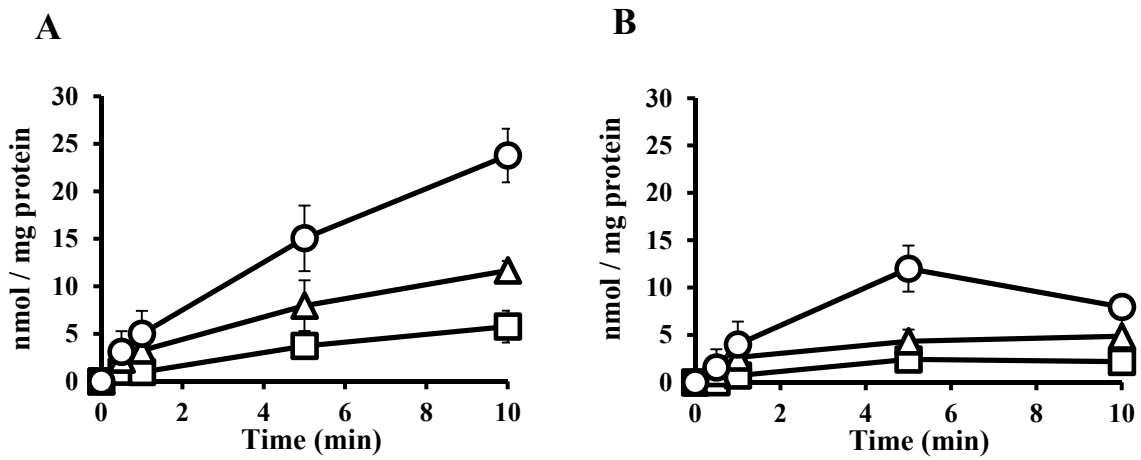


Figure 3-1. Accumulation of [^{3}H]L-alanine (60 Ci/mmol) in the inverted membrane vesicles prepared from a cysteine-less mutant under a downhill solute potential. Inverted membrane vesicles were prepared from MLA301Δ*alaE*/pC22A-28A-135A and incubated with (A) and without (B) 2.5 mM NADH. Extravesicular L-alanine concentrations were set to (□), 50 mM; (△), 100 mM; and (○), 200 mM. Values presented are the means of three independent experiments with standard deviations.

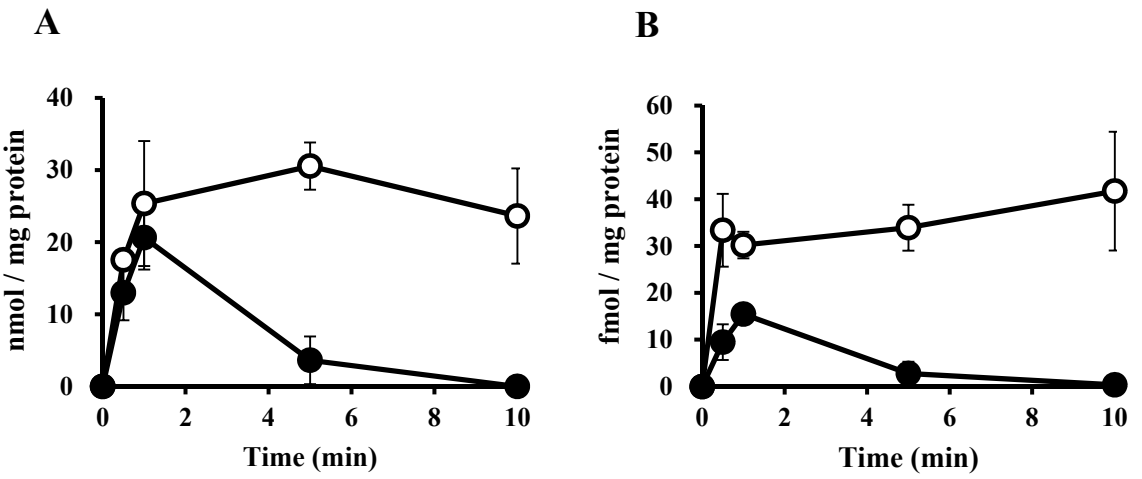


Figure 3-2. Accumulation of [^{3}H]L-alanine (60 Ci/mmol) in the inverted membrane vesicles prepared from the cysteine-less mutant under an equilibrium state and an uphill L-alanine potential. Inverted membrane vesicles were prepared from MLA301Δ*alaE*/pC22-28-135A and loaded with 200 mM L-alanine. The extravesicular L-alanine was set to 200 mM (A), and 0.34 μM (B). Symbols: ○, 2.5 mM NADH; ●, without NADH. Values presented are the means of three independent experiments with standard deviation

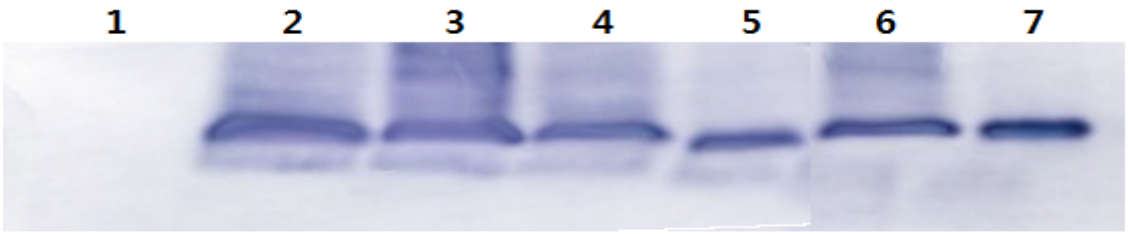


Figure 3-3. SDS Polyacrylamide gel electrophoretogram of the membrane fraction prepared from the wild-type and various AlaE variants tagged with hexahistidine. Membrane fractions equivalent to 1 μg protein were subjected to SDS polyacrylamide gradient gel (10 to 20%) electrophoresis and the protein band was visualized with anti-hexahistidine mouse monoclonal antibody. Lane 1, MLA301Δ*alaE*; lane 2, MLA301Δ*alaE*/pAlaE -His; lane 3, MLA301Δ*alaE*/pC22A-His; lane 4, MLA301Δ*alaE*/pC28A-His; lane 5, MLA301Δ*alaE*/pC135A-His; lane 6, MLA301Δ*alaE*/pC22A-28A-135A-His; and lane 7, Ni-NTA resin-purified hexahistidine-tagged AlaE (0.1 μg protein).

IV. Effects of mutation in charged amino acid residues present in the transmembrane regions of L-alanine exporter AlaE on their activity and growth of cells expressing an individual mutant *alaE* gene

1. Introduction

When Hori isolated L-alanine exporter-less mutants using the peptide feeding method, he obtained twenty-two independent Ala-Ala-hypersentitive mutants by a random chemical mutagenesis experiment from the L-alanine non-metabolizing *E. coli* strain, MLA301 (27). Subsequently, two representative mutants, LAX12 and LAX16, have been found to possess a point mutation in the *alaE* gene (formerly *ygaW* gene), guanine-to-adenine at position 374 and cytosine-to-thymine at position 101 from the initiation codon of *alaE* leading to the amino acid change, glycine-to-glutamic acid (G125E) and serine-to-phenylalanine (S34F), respectively. It is of noted that both mutations were predicted to locate in the transmembrane segments, G125E (LAX12) in TM4 and S34F (LAX16) in TM1, suggesting that these TMs play an important role in AlaE function. The results are in line with the notion that transmembrane regions of transporters are important for helix packing to constitute substrate binding site(s) and energy coupling (50, 51). In particular, charged amino acid residues in the TMs of integral membrane proteins are well-known to play a strikingly pivotal role in their function (52). I therefore focused on three charged amino acid residues present in the predicted TMs of AlaE: Glu at position 30 in TM1 (E30), Arg in TM2 (R45), and Asp in TM3 (D84), and mutagenized respective charged amino acid residues. Subsequently, I evaluated the impacts of each mutation on their activity of the AlaE variants.

2. Materials and methods

2-1) Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 4-1. Cells were grown aerobically at 37°C in L-broth containing 1% tryptone, 0.5% yeast extracts, and 0.5% NaCl (pH 7.2) or minimal medium (30) containing 22 mM glucose, 7.5 mM $(NH_4)_2SO_4$, 1.7 mM $MgSO_4$, 7 mM K_2SO_4, 22 mM NaCl and 100 mM sodium phosphate (pH 7.1). D-alanine (50 μg/ml), L-alanine (50 μg/ml), gentamicin (6.25 μg/ml), kanamycin (6.25 μg/ml), and chloramphenicol (12.5 μg/ml) were supplemented as needed. Growth was monitored by reading the turbidity at 660 nm (A_{660}).

2-2) Direct sequencing of the alaE gene of Ala-Ala-hypersensitive mutants

Twenty LAX series mutants were grown in L-broth containing 50 μg/ml D-alanine at 37°C for overnight. Fully-grown cells were collected by centrifugation (11,096 x g, 4°C), washed once with distilled water, and resuspended in the original volume of distilled water. After boiling the cells suspension for 5 min, the supernatants were obtained by centrifugation as described above and used as a template for the following PCR with primer sets: 5'-CGGGATCCCGTTACCTCACCCCCAAAC-3' and 5'-CGGGATCCCGCGAATGGGACGTACCG-3'. The nucleotide sequences of the *alaE* gene of the LAX series mutants were determined by the dideoxy chain termination method (34).

2-3) Site-directed mutagenesis and construction of mutants

Negatively charged amino acid residues in the TMs of AlaE were changed to cysteine or their respective conserved charged residues by the inverse PCR method using primers with a mismatch codon as shown in Table 4-2. For construction of mutant *alaE* genes, each mutagenized *alaE* gene was amplified using a primer set and pAlaE as

a template followed by *Dpn*I digestion. The resulting PCR products were transformed into *E. coli* JM109. Plasmids were isolated from transformants grown on L-agar medium containing chloramphenicol (12.5 μg/ml) and the entire *alaE* gene was sequenced to ensure that no secondary mutation was present in the gene. The *alaE* variants with a hexahistidine at their C-termini were constructed by PCR using the same primer sets as above and pAlaE-His as the template. The hexahistidine-tagged *alaE* genes were sequenced to verify no secondary mutations in the mutagenized *alaE* genes.

2-4) Determination of the minimum inhibitory concentration (MIC) of L-alanyl-L-alanine (Ala-Ala)

The minimum inhibitory concentration of Ala-Ala was determined as a measure of L-alanine transport activity. MLA301Δ*alaE* cells harboring individual *alaE* variant-bearing plasmid were grown in L-broth containing 50 μg/ml D-alanine, 6.25 μg/ml gentamicin, 6.25 μg/ml kanamycin, and 12.5 μg/ml chloramphenicol at 37°C overnight, harvested, washed twice with 0.85% NaCl, and suspended in the same solution. Approximately 1~3 x 10^4 cells per 5 μl were spotted using the Microplanter MIT-P (Sakuma Co., Japan) onto minimal agar medium containing a two-fold serially diluted Ala-Ala (1.25 to 10,000 μg/ml) and incubated at 37°C. MICs were scored after 24 h and 44 h of incubation.

2-5) Monitoring the growth of cells expressing each *alaE* variant gene

The growth of MLA301Δ*alaE* cells harboring a mutant *alaE*-bearing plasmid were grown aerobically in L-broth containing 50 μg/ml D-alanine, 6.25 μg/ml gentamicin, 6.25 μg/ml kanamycin, 12.5 μg/ml chloramphenicol at 37°C overnight. Cells (5 μl) were added to 5 ml of L-broth containing D-alanine (50 μg/ml), gentamicin

(6.25 μg/ml), kanamycin (6.25 μg/ml), and chloramphenicol (12.5 μg/ml). When growth was evaluated in minimal medium, overnight-grown cells in L-broth were harvested by centrifugation at 8,900 x g for 10 min, washed twice with 0.85% NaCl, and resuspend in the original volume of minimal medium. Cells (5 μl) were added to 5 ml of minimal medium containing D-alanine (50 μg/ml), L-alanine (20, 50, or 100 μg/ml), gentamicin (6.25 μg/ml), kanamycin (6.25 μg/ml), and chloramphenicol (12.5 μg/ml). Subsequently, cells were grown aerobically by reciprocal shaking at 37°C and growth was monitored by reading the turbidity at 660 nm (A_{660}).

2-6) L-alanine accumulation in intact cells

Cells were grown in L-broth containing D-alanine (50 μg/ml) and appropriate antibiotics as described above at 37°C overnight, and harvested by centrifugation at 8,900 x g for 10 min. Cells were then washed twice with minimal medium containing D-alanine (50 μg/ml), gentamicin (6.25 μg/ml), and kanamycin (6.25 μg/ml) and suspended in the same medium adjusting the cell density to A_{660} 0.3. After incubation at 37°C for 3 h, cells were collected by centrifugation as described above, suspended in the same ice-cold minimal medium adjusting the cell density to A_{660} 1.0, and kept on ice until use. A plastic tube containing 1.5 ml of cell suspension in the presence of 300 μg/ml of chloramphenicol was pre-incubated at 37°C for 10 min. The reaction was initiated by adding 750 μl of the same minimal medium containing 20 μM L-[2,3-^{3}H]alanine (38 Ci/mmol, Moravek Biochemicals, USA) and terminated by filtering 100 μl aliquots through a membrane filter with a pore size of 0.22 μm followed by washing twice with 3 ml each of pre-warmed medium. A membrane filter was placed in a vial and immersed in 6 ml of the scintillant Filter-Count (PerkinElmer, USA). The radioactivity was counted after 24 h by the liquid scintillation counter, SLC-5001 (Hitachi Aloka Medical, Japan).

2-7) Preparation of the membrane fraction and immunoblotting analysis

Transformants harboring individual mutant *alaE*-bearing plasmid were grown in L-broth containing 50 μg/ml D-alanine and appropriate antibiotics. Cells were harvested, washed twice with 50 mM Tris-HCl (pH 8.0), and resuspended in the same buffer containing 1 mM phenylmethanesulfonyl fluoride (Wako Pure Chemical Industry, Japan). The cell suspension was subjected to sonic oscillation by 15-sec exposure and 45-sec intermittent cooling in an ice-bath at the maximum output using a Bioruptor (model UCD-250, CosmoBio Co., Japan) until the cell suspension became translucent. The sonic extracts were centrifuged at 15,000 x g for 20 min at 4°C to remove unbroken cell debris and the supernatant fraction was centrifuged again at 100,000 x g for 1 h at 4°C. The resulting membrane pellets were suspended in phosphate-buffered saline (pH 7.4) (49), mixed with the electrophoresis sample buffer and heated in a boiling water bath for 5 min (33). The membrane fraction containing 0.2 μg proteins was subjected to SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane (Immobion-PVDF, Millipore, USA) using a blotting apparatus (model WSE-4020, ATTO, Japan), and probed with mouse monoclonal antibody raised against hexahistidine (Wako Pure Chemical Industries, Japan).

3. Results

3-1) Ala-Ala-hypersensitive mutants have a mutant *alaE* gene with an amino acid change in the predicted transmembrane segments

Since Ala-Ala-hypersensitive mutants LAX12 and LAX16 were found to have a point mutation, G125E and S34F, in the transmembrane segment TM4 and TM1, respectively, the rest of the Ala-Ala-hypersensitive mutants were also expected to possess a mutation(s) in the *alaE* gene. I therefore determined the nucleotide sequence of the *alaE* gene of the other twenty Ala-Ala-hypersensitive mutants (Table 4-3). Out

of twenty mutants, three mutants, LAX9, LAX10, and LAX21, were found to possess a point mutation(s) in the *alaE* gene resulting in an amino acid change S34F, S116L, and G129D, respectively. Interestingly, LAX9 had a silent mutation, cytosine-to-thymine at position 426 from the initiation codon, besides a missense mutation S34F, indicating that LAX9 and LAX16 were independent clones. In addition, LAX4 was found to have a guanine-to-adenine mutation at nucleotide position 234, leading to a premature stop codon. Notedly, all these amino acid substitution were present in the predicted TM1 (S34F) and TM4 (S116L, G129D), suggesting that these TMs are important for L-alanine export activity. In addition, LAX1 and LAX3 were found to have the same nucleotide change, thymine-to-adenine, at position -88 from the initiation codon. Since this mutant had no amino acid change in the open reading frame of AlaE and the nucleotide change were present in the putative -10 region (27), it was suggested that the nucleotide change in LAX1 and LAX3 could result in a reduction of the *alaE* expression.

3-2) Impacts of the mutation of charged amino acid residues in the predicted transmembrane segments of AlaE on the MIC of Ala-Ala

To evaluate the impacts of the mutation of charged amino acid residues, glutamic acid-30, arginine-45, and aspartic acid-84, present in the predicted TM1,TM2, and TM3, respectively, I determined the MIC of Ala-Ala for MLA301Δ*alaE* harboring the individual recombinant plasmid pE30C, pE30D, pR45C, pR45K, pD84C, and pD84E (Table 4-4). Replacement of aspartic acid at position 84 with cysteine (D84C) exerted striking effect on the susceptibility of MLA301Δ*alaE* toward Ala-Ala showing MIC of 78 μg/ml at 44h of incubation. In contrast, conserved amino acid change, aspartic acid-to-glutamic acid (D84E), rendered Ala-Ala resistance to MLA301Δ*alaE*

cells to the same level as that of wild-type *alaE*, suggesting that negative charge at this position is important for AlaE function.

When glutamic acid-30 was replaced with cysteine (E30C), MLA301Δ*alaE* expressing this AlaE variant showed MICs of 20 μg/ml and >10 mg/ml at 24 h and 44 h, respectively, of incubation, indicating that the mutation reduced its activity to some extent. Interestingly, however, glutamic acid-to-aspartic acid mutation at this position, E30D, had severer effect on the AlaE activity than that of E30C since the conserved mutation exhibited MICs of 10 μg/ml and 156 μg/ml at 24 h and 44 h, respectively, of incubation, suggesting that negative charge at position 30 might not be important for AlaE function compared with the importance of the negatively charged residue at position 84.

Of noted, the mutation of arginine at position 45, among three charged residues, exerted the most pronounced effect on the Ala-Ala susceptibility showing that the MICs for MLA301Δ*alaE* expressing AlaE variant, R45C and R45K, were nearly the same as those in *alaE*-deficient MLA301Δ*alaE* and its pSTV29 (empty vector)-bearing transformant (Table 4-3). The results suggested that arginine-45 plays an important role in the AlaE function.

3-3) Accumulation of [^{3}H]L-alanine in intact cells

Since mutation of the charged amino acid residues in the TM regions showed various levels of impact on AlaE activity (Table 4-4), I asked whether the MIC results reflect accumulation of [^{3}H]L-alanine in intact cells expressing each *alaE* variant gene. MLA301Δ*alaE* expressing either glutamic acid-30 variant, E30C or E30D, accumulated 3.8 or 3.2 nmol L-alanine/mg dry weight, respectively, the levels of which were between the uptake levels in MLA301Δ*alaE* and MLA301Δ*alaE*/pAlaE overexpressing the wild-type *alaE* gene (Fig. 4-1A). The results are generally consistent with the MIC

results where both mutant AlaE, E30C and E30D, showed lower MIC values than the wild-type AlaE (Table 4-3).

MLA301Δ*alaE* expressing AlaE variant D84E accumulated a quite small amount of [^{3}H]L-alanine, level of which were almost the same as that obtained in cells expressing the wild-type AlaE (Fig. 4-1C). On the other hand, the host cells expressing the AlaE variant D84C accumulated a similar level of [^{3}H]L-alanine as that in MLA301Δ*alaE*. These results were in good agreement with the MIC results as described above.

I observed quite intriguing results with arginine-45 variants, R45C and R45K (Fig. 4-1B). Although MLA301Δ*alaE*/pR45C expressing arginine-to-cysteine AlaE mutant accumulated almost the same level of [^{3}H]L-alanine as that in MLA301Δ*alaE*, which is consistent with the significantly low MIC values toward Ala-Ala, <1.25 μg/ml and 10 μg/ml at 24 h and 44 h, respectively, of incubation. Interestingly, however, MLA301Δ*alaE* expressing AlaE variant, R45K, accumulated much lower and slightly higher levels of [^{3}H]L-alanine than MLA301Δ*alaE* and MLA301Δ*alaE*/pAlaE, respectively (Fig. 4-1B), although R45K showed almost nil activity as assessed by MIC determination (Table 4-4). Intracellular accumulation of [^{3}H]L-alanine is a sum of the uptake and excretion of L-alanine. Therefore, the results for the AlaE variant, R45K, obtained in the [^{3}H]L-alanine uptake experiment strongly suggested that the mutant AlaE (R45K) retained at least a partial L-alanine export activity.

3-4) Expression of the AlaE variants tagged with hexahistidine

MLA301Δ*alaE* expressing the AlaE mutant, R45C and R45K, showed almost the same MIC of Ala-Ala as those in *alaE*-deficient host cells and MLA301Δ*alaE* harboring an empty vector (Table 4-4). One may argue, therefore, that replacement of the arginine residue at position 45 with cysteine or lysine reduces the expression level of

each AlaE variant, which in turn affects susceptibility of the transformants toward Ala-Ala. To clarify this ambiguity, I constructed hexahistidine-tagged *alaE* genes at their C-terminal ends. When the activity of the respective histidine-tagged AlaE variants was assessed by determining the MIC of Ala-Ala for MLA301Δ*alaE* expressing either AlaE variant, R45C or R45K, all histidine-tagged AlaE mutants showed levels of MIC similar to those obtained with their respective histidine-less AlaE variants except for R45K (Data not shown). Next, I isolated membrane fractions from cells expressing each histidine-tagged AlaE variant and analyzed their expression levels by the western blot analysis, which was subsequently probed with the anti-hexahistidine mouse monoclonal antibody. The expression of AlaE variants except for R45C appeared to be comparable with that of the wild-type AlaE (Fig. 4-2). Although the expression level of R45C was lower than that of wild-type AlaE (Fig 4-2, lanes 3 and 6), still, R45C protein band was detected.

3-5) The effect of arginine-45 mutation on growth of MLA301Δ*alaE* expressing AlaE variant R45C or R45K

Since the *alaE* mutant possessing arginine-to-lysine mutation at position 45 showed a unique phenotype, that is, MLA301Δ*alaE*/pR45K exhibited a supersusceptibility to Ala-Ala (Table 4-4), but accumulated much less amount of [^{3}H]L-alanine than that in the *alaE*-deficient host cells MLA301Δ*alaE* (Fig. 4-1B). Thus, I hypothesized that the hypersusceptibility of MLA301Δ*alaE*/pR45K toward Ala-Ala might be due to its poor growth property under the MIC determination conditions. Accordingly, I evaluated the effect of mutation at position 45 on growth of MLA301Δ*alaE* expressing each AlaE variant, R45K and R45C, in minimal liquid medium. As shown in figure 4-3A MLA301Δ*alaE*/pR45C showed a growth pattern similar to that of MLA301Δ*alaE* and MLA301Δ*alaE*/pAlaE expressing the wild-type

AlaE, although a maximum level of growth of MLA301Δ*alaE*/pR45C was slightly lower than that of MLA301Δ*alaE* with or without pAlaE. Notably, growth retardation of MLA301Δ*alaE*/pR45K in minimal medium containing D-alanine (50 μg/ml) and L-alanine (20 μg/ml) was reproducibly observed, indicating that the extremely low MIC value of Ala-Ala for this strain had probably caused by its poor growth under the conditions tested.

It is of great interest to note that, when MLA301Δ*alaE* expressing either AlaE variant, R45C or R45K, was grown in L-broth containing 50 μg/ml D-Ala, MLA301Δ*alaE*/pR45K grew much better than MLA301Δ*alaE*/pR45C, a level of which was comparable to that of wild-type AlaE (Fig. 4-3B). I therefore speculated that certain component(s) present in L-medium might influence the growth of MLA301Δ*alaE* expressing the AlaE variants R45C and R45K. Since L-alanine is a major substrate of AlaE (27), I evaluated the effect of L-alanine on growth of *alaE*-deficient host cells expressing the AlaE variants on minimal medium (Fig. 4-4). MLA301Δ*alaE* expressing the wild-type AlaE grew better on minimal agar medium containing 100 μg/ml L-alanine than on the some medium containing 20 μg/ml L-alanine (Fig. 4-4A and B). However, the growth of cells expressing the AlaE variant R45C on minimal agar medium with 100 μg/ml L-alanine was slightly poorer than that on minimal medium containing 20 μg/ml L-alanine (Fig. 4-4A and B). In addition, this negative impact of L-alanine on growth levels of MLA301Δ*alaE*/pR45C was inversely correlated with the amount of L-alanine supplemented in minimal medium (Fig. 4-4C). In addition, *alaE*-deficient cells expressing AlaE mutant R45K also showed a similar pattern of growth response as that obtained with R45C on minimal medium (Fig. 4-4A and B).

4. Discussion

It is well known that transmembrane helices of transporters play a pivotal role for their function and tertiary structure formation in the membranes, which, in general, are composed of hydrophobic amino acid residues of about 20 residues in length (53). Presence of a single charged amino acid residue in the transmembrane region is thermodynamically unfavorable, hence, the charged amino acid residue(s) is especially of importance to perform their function, such as substrate binding, energy coupling, and salt bridge formation (50, 51, 54). I therefore focused in this study on three charged amino acid residues, E30, R45, and D84, present in the predicted transmembrane segments of AlaE and evaluated the effects of amino acid change of each charged residue on their function.

Hori has found that when CCCP, a proton ionophore, was present in an L-alanine production model system in which *E. coli* MG1655 expressed heterologous alanine dehydrogenase (27), L-alanine excretion level was reduced under a stand culture conditions, suggesting that proton motive force might be an energy source for L-alanine transport via AlaE (27). In this respect, glutamic acid-30 is unlikely to form a coupling cation translocation pathway since the conserved amino acid change (E30D) showed more detrimental effects on the AlaE function than amino acid replacement from glutamic acid to cysteine (Table 4-4). On the other hand, aspartic acid-to-glutamic acid change at position 84 fully retained L-alanine export activity of AlaE, but the cysteine variant (D84C) strikingly reduced its activity (Table 4-4, Fig. 4-1). A pivotal role(s) of acidic amino acid residues in the transmembrane segments has well been documented in several ion-coupled transporters: residues involved in i) substrate binding are multidrug transporter MdfA (55, 56) and tetracycline transporter TetB (57) in *E. coli*, multidrug transporter QacA in *Staphylococcus aureus* (58), and lactose permease LacY in *E. coli* (59, 60, 61, 62); ii) binding and translocation of co-

transported cation are lactose permease LacY(59, 60, 61, 62), melibiose transporter MelB (63, 64, 65), and Na^+/H^+ antiporter NhaA (66, 67) in *E.coli*; and iii) either substrate binding and proton translocation in multidrug antiporter EmrE (52, 54) and lactose permease LacY (62, 68) in *E. coli*. Thus, I interpreted the results of amino acid change at position 84 to mean that negative charge at this position plays an important role in AlaE function, probably proton coupling and/or substrate binding.

In view of positively charged residues, arginine-144 of LacY, the most thoroughly investigated secondary transporter, appears to involve in substrate binding and salt-bridge (69, 70). The LacY mutant R144K, a conservative mutation, reduces the lactose accumulating activity to a level of about 25% of wild-type LacY, whereas R144C mutant shows almost no activity (69). In analogy with this, it is tempting to speculate that arginine-45 of AlaE might be involved in substrate binding. In this respect, it is interesting to note that growth response of AlaE mutants, R45C and R45K, in minimal medium and rich medium was totally different (Fig. 4-3), where the onset of growth in R45K-expressing cells in minimal medium retarded when compared to that of R45C-expressing cells. Their growth response was completely reversed in rich medium (Fig. 4-3). Growth of R45K in L-broth was nearly the same as those of MLA301Δ*alaE* and MLA301Δ*alaE*/pAlaE expressing the wild-type AlaE, whereas growth of R45C-expressing cells was slower than R45K-expressing cells (Fig. 4-3). L-broth differs from minimal medium in the contents of various nutrients including L-alanine. I thus asked whether supplementation of L-alanine (20 μg/ml and 100 μg/ml) in minimal medium influences the growth of MLA301Δ*alaE* expressing the AlaE variants, R45C and R45K. Notably, growth of *alaE*-deficient cells expressing R45C and R45K on minimal medium containing 100 μg/ml L-alanine was slower than that on the same medium containing 20 μg/ml L-alanine (Fig. 4-4), suggesting that high concentration of L-alanine may affect the functionality of this mutant AlaE.

Taken together, these results are in line with the hypothesis that argine-45 could form a substrate binding site of AlaE. Further biochemical and molecular genetic analyses will be needed for more in-depth understanding in the molecular mechanism by which AlaE exports L-alanine.

Table 4-1. List of strains and plasmids used

Strain or plasmid	Relevant properties*	Source or reference
Escherichia coli		
JM109	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17(r_k^- m_k^+), *e*14$^-$(*mcrA*$^-$), *supE*44, *relA*1, Δ(*lac-proAB*)/F'[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	Laboratory strain
MLA301	MG1655 *alr*::*FRT*, *dadX*::*FRT*, *yfdZ*::*FRT*, *avtA*::GM, yfbQ::KM	This study
MLA301Δ*alaE*	MLA301 with a deletion in the *alaE* gene	This study
LAX1-22	MLA301 derivative hypersensitive to Ala-Ala	(27, 29)
Plasmid		
pSTV29	CP^r, *lacZ*, p15A ori	Takara
pAlaE	pSTV29 harboring 1.0-kb PCR fragment of the *alaE* gene	(27, 28)
pE30C	pAlaE derivative with Glu replacement at position 30 with Cys	This study
pE30D	pAlaE derivative with Glu replacement at position 30 with Asp	This study
pR45C	pAlaE derivative with Arg replacement at position 45 with Cys	This study
pR45K	pAlaE derivative with Arg replacement at position 45 with Lys	This study
pD84C	pAlaE derivative with Asp replacement at position 84 with Cys	This study
pD84E	pAlaE derivative with Asp replacement at position 84 with Glu	This study
pAlaE-His	pAlaE derivative possessing hexa histidine at the C-terminal of the *alaE* gene	This study
pE30C-His	pE30C derivative possessing hexa histidine at the C-terminal of the *alaE* gene	This study
pE30D-His	pE30D derivative possessing hexa histidine at the C-terminal of the *alaE* gene	This study

pR45C-His	pR45C derivative possessing hexa histidine at the C-terminal of the *alaE* gene	This study
pR45K-His	pR45K derivative possessing hexa histidine at the C-terminal of the *alaE* gene	This study
pD84C-His	pD84C derivative possessing hexa histidine at the C-terminal of the *alaE* gene	This study
pD84E-His	pD84E derivative possessing hexa histidine at the C-terminal of the *alaE* gene	This study

*Abbreviations for antibiotics: GM, gentamicin; KM, kanamycin; CP, chloramphenicol.

Table 4-2. Primers used in this study

Primer	Nucleotide sequence*	Codon change
E30C-F	5'-TGTATTTGTGTTTTCCTCTCCGGAATGAG-3'	GAA→TGT
E30C-R	5'-GAAAACACAAATACACATGTTCACGACAG-3'	TTC→ACA
E30D-F	5'-TGTATTGATGTTTTCCTCTCCGGAATGAG-3'	GAA→GAT
E30D-R	5'-GAAAACATCAATACACATGTTCACGACAG-3'	TTC→ATC
R45C-F	5'-TTTATTCCTGTTTGGTAGCGATTCCGGTG-3'	AGA→TGT
R45C-R	5'-CCAAACAGGAATAAAAAGACTGTTCGAAGC-3'	TCT→ACA
R45K-F	5'-TTTATTCCAAATTGGTAGCGATTCCGGTG-3'	AGA→AAA
R45K-R	5'-CCAATTTGGAATAAAAAGACTGTTCGAAGC-3'	TCT→TTT
D84C-F	5'-ATCTGGCGTGTATCCTGGCTTATGTGACG-3'	GAT→TGT
D84C-R	5'-GGATACACGCCAGATTTTTTATCCAGCCCG-3'	ATC→ACA
D84E-F	5'-ATCTGGCGGAAATCCTGGCTTATGTGACG-3'	GAT→GAA
D84E-R	5'-GGATTTCCGCCAGATTTTTTATCCAGCCCG-3'	ATC→TTC

*, Underlined triplet codons represent mutagenized codons

Table 4-3. Results of nucleotide sequencing of the *alaE* gene in the Ala-Ala-hypersensitive mutants

Strain[a]	Nucleotide change in the *alaE* gene[b]	amino acid change[c]	location of the mutation	reference
LAX1	T→C (-88)		-10 region	This study
LAX3	T→C (-88)		-10 region	This study
LAX4	G→A (234)	Trp→Stop (78)	Loop II/III	This study
LAX9	C→T (101)	Ser→Phe (34)	TM1	This study
	C→T (426)	Ser→Ser (142)	C-terminal (cytoplasmic)	This study
LAX10	C→T (347)	Ser→Leu (116)	TM4	This study
LAX12*	G→A (374)	Gly→Glu (125)	TM4	(27)
LAX16*	C→T (101)	Ser→Phe (34)	TM1	(27)
LAX21	G→A (386)	Gly→Asp (129)	TM4	This study

*Data are taken from reference (Hori, Ph.D. thesis, 2011)

[a]The rest of 14 LAX seriese Ala-Ala-hypersensitive mutants have no mutation in the *alaE* gene.

[b]The numbers in parentheses are the nucleotide changed from the initiation codon.

[c]The numbers in parentheses are the position of amino acid residue changed from the initiation codon.

Table 4-4. Minimum inhibitory concentration (MIC) of L-alanyl-L-alanine (Ala-Ala) for *alaE*-deficient cells expressing various AlaE variants

Strain	MIC (μg/ml)	
	24 h	44 h
MLA301	156	>10,000
MLA301Δ*alaE*	<1.25	2.5
MLA301Δ*alaE* /pAlaE	>10,000	>10,000
MLA301Δ*alaE* /pstv29	<1.25	<1.25
MLA301Δ*alaE* /pE30C	20	>10,000
MLA301Δ*alaE* /pE30D	10	156
MLA301Δ*alaE* /pR45C	<1.25	10
MLA301Δ*alaE* /pR45K	<1.25	<1.25
MLA301Δ*alaE* /pD84C	10	78
MLA301Δ*alaE* /pD84E	>10,000	>10,000

The MIC was scored after 24 h and 44 h of incubation. The MIC values presented are the results of more than three independent assays.

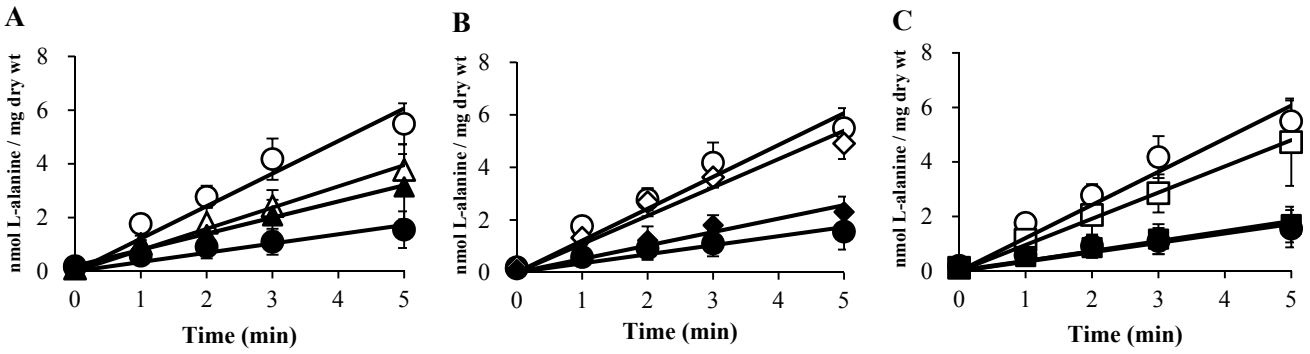


Figure 4-1. Accumulation of [^{3}H]L-alanine in intact cells. Cells were grown in minimal medium to the mid-log phase, washed, and suspended in the same medium. Transport assay was initiated by adding [^{3}H]L-alanine (38 Ci/mmol) to the final concentration of 10 μM and then incubated at 37°C. An aliquot (100 μl) of the cells suspension was filtered through a membrane filter with a 0.22 μm-pore size, and then washed twice with 3 ml of pre-warmed minimal medium. Values presented are the means of three independent experiments with standard deviations. Symbols in A: ○, MLA301Δ*alaE*; ●, MLA301Δ*alaE*/pAlaE; ▵, MLA301Δ*alaE*/pE30C; and ▲, MLA301Δ*alaE*/pE30D. Symbols in B: ○, MLA301Δ*alaE*; ●, MLA301Δ*alaE*/pAlaE; ◊, MLA301Δ*alaE*/pR45C; and ♦, MLA301Δ*alaE*/pR45K. Symbols in C: ○, MLA301Δ*alaE*; ●, MLA301Δ*alaE*/pAlaE; □, MLA301Δ*alaE*/pD84C; and ■, MLA301Δ*alaE*/pD84E.

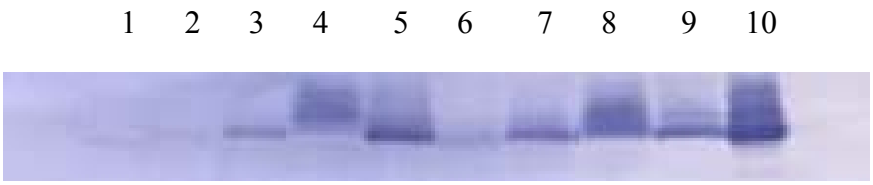


Figure 4-2. Polyacrylamide gel electrophoretogram of the membrane fraction prepared from cells expressing the wild-type and various AlaE variants tagged with hexahistidine. Membrane fractions equivalent to 1 μg protein were subjected to SDS polyacrylamide gradient gel (10 to 20%) electrophoresis and the protein band was visualized with anti-hexahistidine mouse monoclonal antibody. Lane 1, MLA301Δ*alaE*; Lane 2, MLA301Δ*alaE*/pAlaE; Lane 3, MLA301Δ*alaE*/pAlaE-His; Lane 4, MLA301Δ*alaE*/pE30C-His; Lane 5, MLA301Δ*alaE*/pE30D-His; Lane 6, MLA301Δ*alaE*/pR45C-His; Lane 7, MLA301Δ*alaE*/pR45K-His; Lane 8, MLA301Δ*alaE*/pD84C-His; Lane 9, MLA301Δ*alaE*/pD84E-His; and Lane 10, Ni-NTA resin-purified histidine-tagged AlaE (100 ng protein).

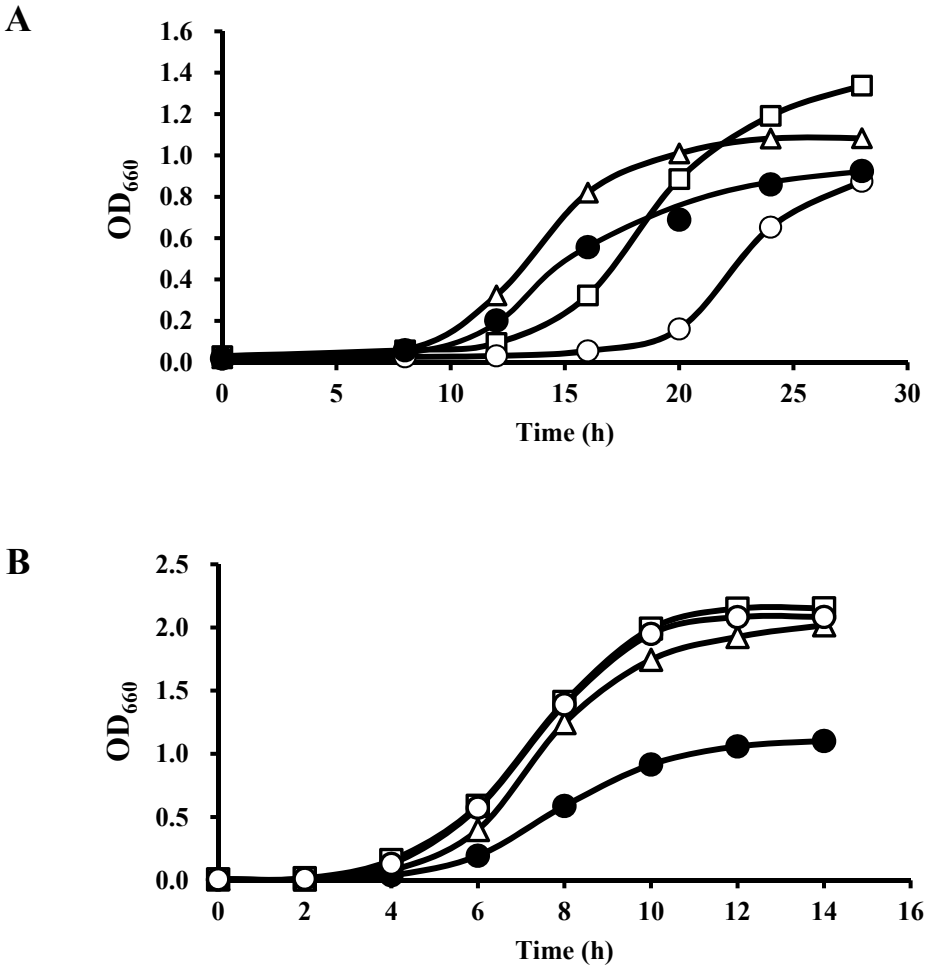


Figure 4-3. **(A)** Growth of AlaE variant-expressing cells in minimal medium containing D-alanine (50 μg/ml), GM (6.25 μg/ml), KM (6.25 μg/ml), CP (12.5 μg/ml) and L-alanine (20 μg/ml). **(B)** Growth of AlaE variants in L-broth containing D-alanine (50 μg/ml), GM (6.25 μg/ml), KM (6.25 μg/ml), and CP (12.5 μg/ml). Symbols: **▲,** MLA301Δ*alaE*/pAlaE; **□,** MLA301Δ*alaE*; **●,** MLA301Δ*alaE*/pR45C; **○,** MLA301Δ*alaE*/pR45K

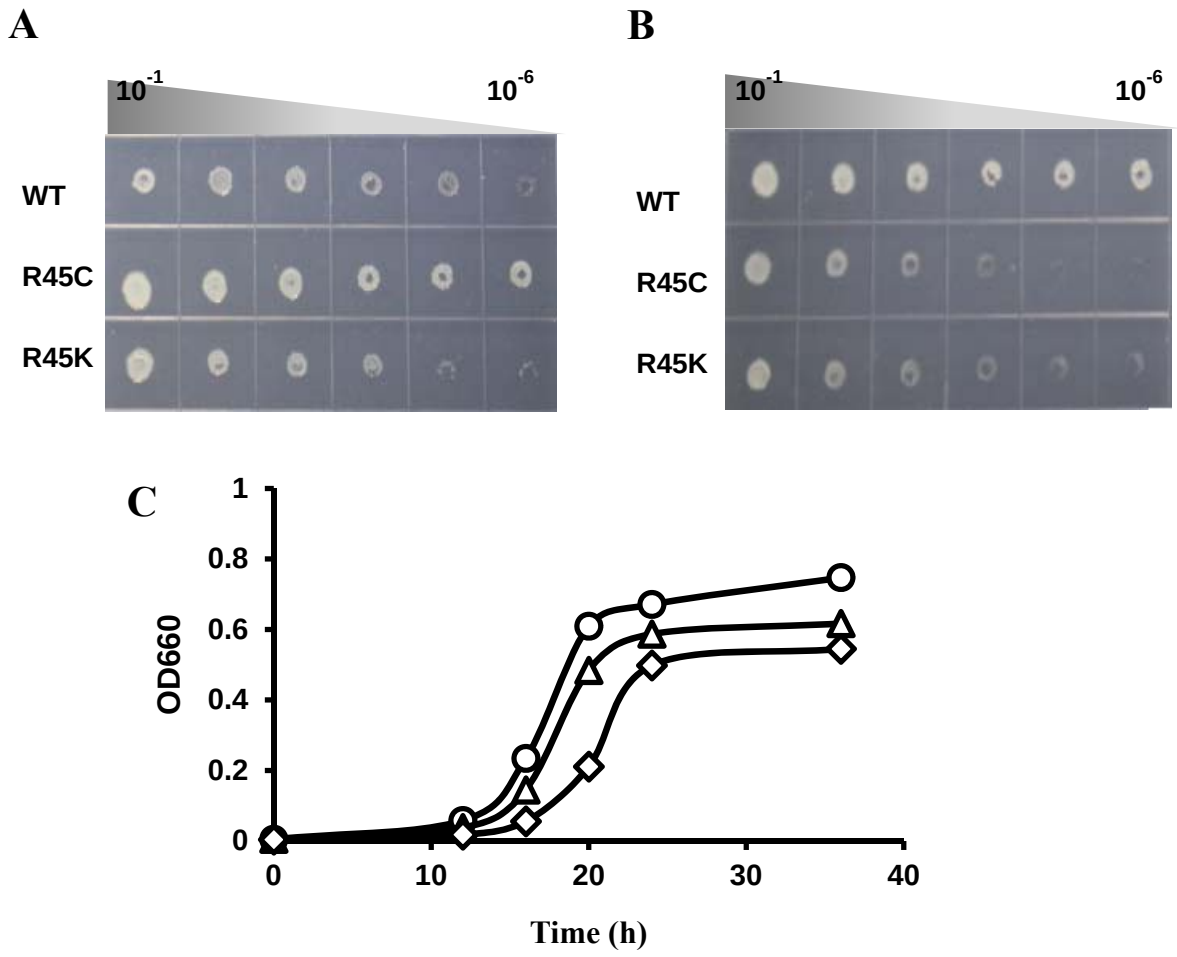


Figure 4-4. **(A)** Growth of AlaE variant-expressing cells in minimal medium plate containing D-alanine (50 μg/ml), GM (6.25 μg/ml), KM (6.25 μg/ml), CP (12.5 μg/ml) and L-alanine (20 μg/ml). **(B)** Growth of AlaE variants in minimal medium plate containing D-alanine (50 μg/ml), GM (6.25 μg/ml), KM (6.25 μg/ml), CP (12.5 μg/ml) and L-alanine (100 μg/ml). **(C)** Growth of AlaE variant (R45C)-expressing cells in minimal medium containing D-alanine (50 μg/ml), GM (6.25 μg/ml), KM (6.25 μg/ml), CP (12.5 μg/ml) and L-alanine. Symbols: ○, 20 μg/ml L-alanine; ∆, 50 μg/ml L-alanine; and ◊, 100 μg/ml L-alanine.

V. Conclusion

The results obtained with the inverted membrane vesicles demonstrate that AlaE catalyzes active export of L-alanine using electrochemical potential of proton as an energy source. To pursue the molecular mechanisms of L-alanine transport via AlaE, further in-depth biochemical and structural analyses are required. Thus, I constructed in this study the cysteine-less AlaE variant and found that it retains L-alanine transport activity although the activity is slightly lower than that of wild-type AlaE.

In addition, I focused on three charged amino acid residues present in the predicted transmembrane segments, Glu30 (TM1), Arg45 (TM2), and Asp84 (TM3), and constructed their AlaE variants. Although subsequent analyses with intact cells expressing individual mutant AlaE of these charged residues are still premature to draw a concrete conclusion, it is tempting to speculate that aspartic acid 84 and arginine 45 might be involved in an energy coupling, probably a cation translocation, and substrate recognition, respectively.

Taken together, this study establishes a foundation for future in-depth structural and functional analyses of AlaE. Cysteine-less AlaE constructed in this study will serve a powerful tool for such experiments.

VI. Reference

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