Supporting Information

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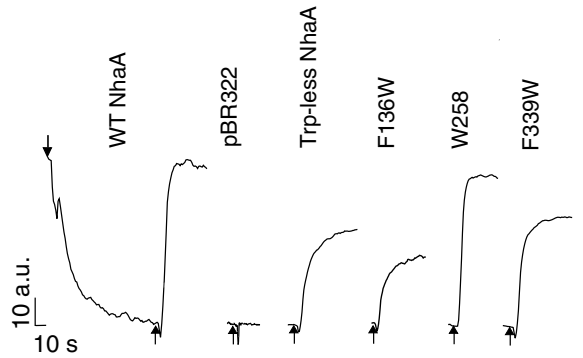

Fig. S1. Li^+/H^+ antiporter activity in everted membrane vesicles. The Li^+/H^+ antiporter activity was determined in everted membrane vesicles isolated from EP432, an *Escherichia coli* strain that lacks the two Na^+/H^+ antiporters (NhaA and NhaB), cells expressing WT NhaA, Trp-less-NhaA, and its single-Trp variants and grown in LBK (Luria broth with KCl instead of NaCl) (pH 7). The ΔpH across the membranes was monitored using the fluorescence of acridine orange. The fluorescence assay was performed with 2.5 mL reaction mixture containing 50–100 μg of membrane protein, 0.5 μM acridine orange, 150 mM KCl, 50 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane, 5 mM $MgCl_2$, and the pH was titrated with HCl. The data of typical experiments are shown. At the onset of the reaction, D-lactate (2 mM) was added (↓) and the fluorescence quenching (*Q*) was recorded until a steady-state level of ΔpH (100 % quenching) was reached. LiCl (10 mM) was then added (↑), and the new steady state of fluorescence obtained (dequenching) was monitored. Fluorescence dequenching indicated that protons are exiting the vesicles in response to Na^+ or Li^+ influx via the antiporter. All experiments were repeated at least three times with practically identical results.

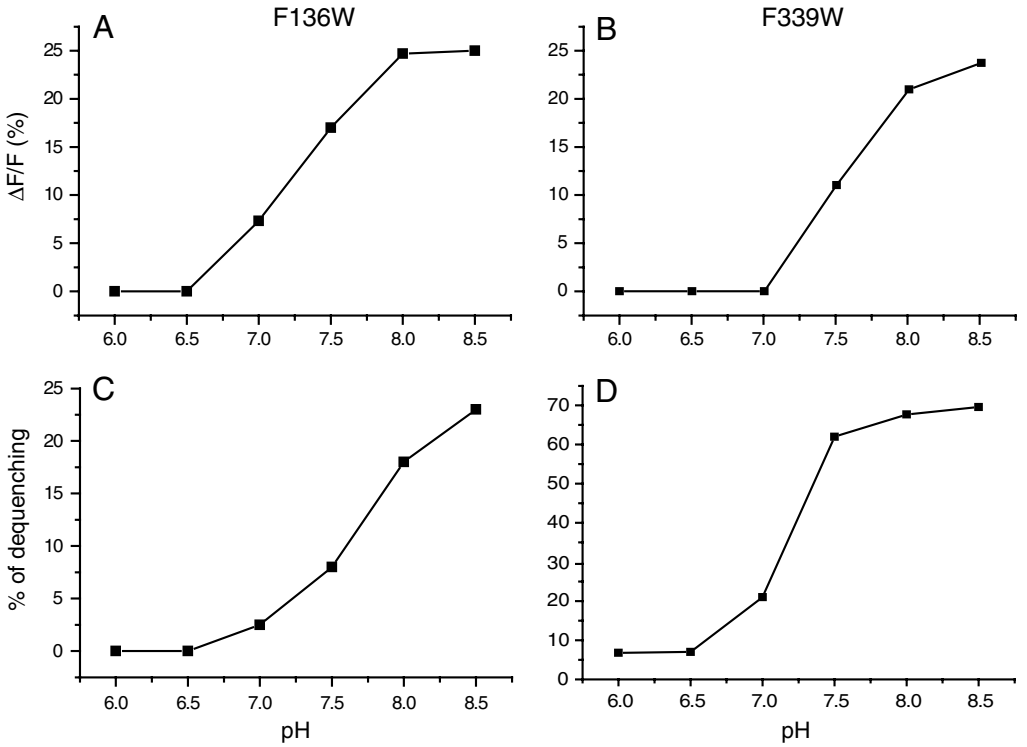

Fig. S2. The pH dependence of the fluorescence responses of single Trp/F136W and single Trp/F339W as compared to the pH profile of their antiport activity in membrane vesicles. (*A*) Single Trp-F136W protein was incubated in a reaction mixture described in Fig. 2 at pH 6 and the pH was stepwise (0.5 unit) increased to pH 8.5. At each pH step, the emission at 338 nm was determined and the dequenching increments were plotted as a function of pH. (*B*) Reaction mixtures with single Trp/F339W protein were prepared as described in Fig. 2 at different pH values as indicated. Li^+ (10 mM) was added to each reaction mixture and the fluorescence changes were monitored at 335 nm. (*C* and *D*) The pH dependence of the Na^+/H^+ or Li^+/H^+ antiporter activity of (*C*) single Trp/F136W and (*D*) single Trp/F339W as measured in isolated membrane vesicles by acridine orange assay with 10 mM NaCl as described in Fig. 1*B*.

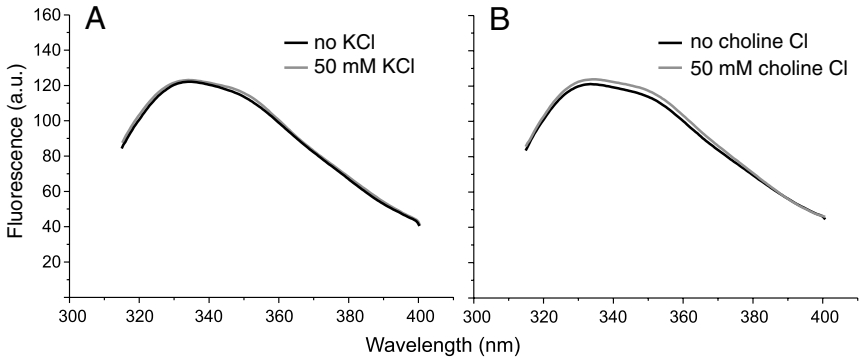

Fig. S3. The ligand-induced fluorescent change in single Trp/F339W is highly specific. The emission spectra of single Trp/F339W was measured in a reaction mixture as in Fig. 2 at pH 8.5 in the presence (gray) or absence (black) of 50 mM KCl (*A*) or 50 mM choline chloride (*B*). The excitation was at 290 nm.

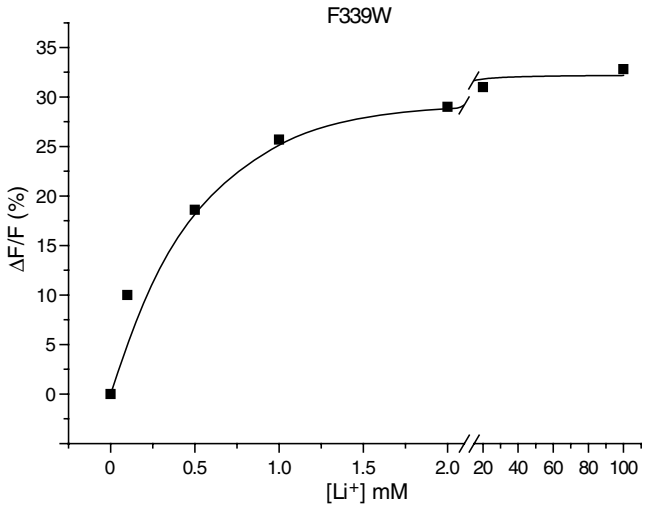

Fig. S4. The dependence of the fluorescence changes of single Trp/F339W on Li^+ concentration. The fluorescence intensities of single Trp/F339W were measured at pH 8.5 in the presence of 0.1–100 mM LiCl. The data were analyzed in Origin 8.0 software assuming that, at saturation, 1 mol of ligand is bound per mole of NhaA.

Table S1. Plasmid variants and growth phenotypes

No.	Plasmid bearing NhaA variants	Plasmid (no.) used as a mutagenesis template	Growth		
			Na^+ (7)	Na^+ (8.2)	Li^+ (7)
1	pAXH3-W37F	26	+++	ND	+++
2	pAXH3-W62C		+++	+++	+++
3	pAXH3-W126F	26	+++	ND	+++
4	pAXH3-W216F	26	+++	ND	+++
5	pAXH3-W258C		+++	+++	+++
6	pAXH3-W309F	26	+++	ND	+++
7	pAXH3-W379F	26	+++	ND	+++
8	pAXH3-W62C-W258C		+++	+++	+++
9	pAXH3-W37F-W62C-W258C	8	+++	ND	+++
10	pAXH3-W37F-W62C-W126F-W258C	9	+++	ND	+++
11	pAXH3-W37F-W62C-W258C-W360F	9	+++	ND	+++
12	pAXH3-W37F-W62C-W126F-W258C-W360F	11	+++	ND	+++
13	pAXH3-W37F-W62C-W216F-W258C-W360F	11	+++	ND	+++
14	pAXH3-W37F-W62C-W126F-W258C-W309F-W360F	12	+++	ND	+++
15	pAXH3-W37F-W62C-W126F-W258C-W360F-W379F	12	+++	ND	+++
16	pAXH3-W37F-W62C -W126F-W216F-W258C-W309F-W360F	14	+++	+++	+++
17	p-Trp-less = pAXH3/W37F-W62C -W126F-W216F-W258C-W309F-W360F-W379F	16	+++	+++	+++
18	p-single-Trp/F136W	17	+++	—	+++
19	p-single-Trp/W258	17	+++	+++	+++
20	p-single-Trp/F339W	17	+++	—	+++
21	pAXH3/F136W	26	+++	+++	+++
22	pAXH3/F339W	26	+++	+++	+++
23	p-single-Trp/F136W/D164N	18	—	—	—
24	p-single-Trp/W258/D164N	19	—	—	—
25	p-single-Trp/F339W/D164N	20	—	—	—
26	pAXH3 (positive control)	—	+++	+++	+++
27	pBR322 (negative control)	—	—	—	—

For characterization of the mutated NhaA variant, EP432 cells transformed with the plasmids carrying the indicated mutations were used. Growth was tested on selective plates comprised of LB of which NaCl is replaced by 0.6 M NaCl at pH 7 or 8.2 (indicated in brackets) or 0.1 M LiCl at pH 7. +++, growth similar to the wild type; —, no growth; ND, not determined.

Table S2. Membrane phenotype of Trp-less-NhaA and its single-Trp variants

Trp-less NhaA variants	Expression% of WT	Activity (maximal dequenching %)		Apparent K_M, mM	
		Na^+	Li^+	Na^+	Li^+
Trp-less NhaA	15	48	59	1.7	0.12
Single-Trp/F136W	23	23	31	0.8	0.05
Single-Trp/W258	24	86	95	0.5	0.01
Single-Trp/F339W	18	48	70	2.2	0.12
Single-Trp/F136W/D164N	85	—	—	—	—
Single-Trp/W258/D164N	90	—	—	—	—
Single-Trp/F339W/D164N	8	—	—	—	—
pAXH3 (WT control)	100	97	100	0.15	0.01
pBR322 (negative control)	—	—	—	—	—

For characterization of the NhaA variants, EP432 cells were transformed with the plasmids carrying the indicated mutations or either pAXH3 or pBR322 serving as a positive or negative control, respectively. The protein expression level was expressed as percentage of the WT. The Na^+/H^+ antiporter activity at pH 8.5 was determined in everted membrane vesicles isolated from the respective strains with 10 mM NaCl or LiCl (Fig. S1) and expressed as percentage of dequenching. The apparent K_M for the ions was determined at pH 8.5. As shown previously, the end level of fluorescence dequenching and the concentration of the ion that gives half-maximal dequenching are good estimates of the antiporter activity and the apparent K_M (for Li^+ or Na^+) of the antiporter, respectively. The concentration range of the cations tested was 0.01–100 mM at the indicated pH values and the apparent K_M values were calculated by linear regression of a Lineweaver–Burk plot. Each experiment was conducted at least three times with essentially identical results.