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The Mrp Na^+/H^+ Antiporter Increases the Activity of the Malate:Quinone Oxidoreductase of an *Escherichia coli* Respiratory Mutant

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Mrp catalyzes secondary Na^+/H^+ antiport and was hypothesized to have an additional primary energization mode. Mrp-dependent complementation of nonfermentative growth of an *Escherichia coli* respiratory mutant supported this hypothesis but is shown here to be related to increased expression of host malate:quinone oxidoreductase, not to catalytic activity of Mrp.

The bacterial Mrp antiporter is widely distributed and has important roles in Na^+ and alkali resistance as well as specialized functions in some settings (5, 8). Mrp catalyzes secondary Na^+/H^+ exchange energized by the proton motive force (Δp) (3, 5, 7, 19). The possibility of an additional, primary mode of energization using redox energy was raised (http://saier-144-164.ucsd.edu/tcdb/index.php?tc = 2.A.63) because of the striking sequence similarity between several of the 6–7 hydrophobic proteins encoded by *mrp* operons to membrane-embedded subunits of proton-translocating NADH:quinone oxidoreductases (complex I) of bacteria and mitochondria (3, 13, 14, 20). All *mrp* gene products are required for wild-type antiport levels (5, 8, 10), suggesting unusual complexity for a secondary antiporter. Primary energy coupling would facilitate cytoplasmic pH homeostasis at alkaline pH and low Δp (21). An exergonic reaction could energize Na^+/H^+ antiport with Na^+ effluxed > H^+ taken up, resulting in concurrent $\Delta\Psi$ generation and cytoplasmic H^+ accumulation. Supporting this scenario (7), we found that expression of the *mrp* operon from alkaliphilic *Bacillus pseudofirmus* OF4 caused a four- to fivefold increase in the otherwise poor nonfermentative growth of an NADH dehydrogenase mutant of *Escherichia coli* strain ANN0222 (Δ*nuo* Δ*ndh*) (25). Comparable expression of NhaA, a secondary Na^+/H^+ antiporter of *E. coli*, did not similarly enhance nonfermentative growth of the respiratory mutant (8). We attempted to determine whether the growth complementation by Mrp had a redox basis and, if so, whether this activity was associated with Mrp itself or with a host enzyme.

Mrp-dependent stimulation of oxygen uptake paralleled that of *E. coli* ANN0222 growth on L-lactate (Fig. 1), consistent with a redox basis for the enhanced growth. Previous work showed that Mrp does not confer either NADH dehydrogenase or terminal oxidase activity (3, 7, 8), and spectral studies revealed no difference in the cytochrome contents of membranes from *mrp* and control transformants of *E. coli* ANN0222 (data not shown). Therefore, if complementation is a direct effect of Mrp, then Mrp is likely to possess an activity that increases electron flow via quinone to the host terminal oxidases. The complementation could also arise from an indirect effect caused by enhanced activity of a host respiratory enzyme that increases electron flow to the terminal oxidases. Potential electron donors were screened for their effect on oxygen uptake by everted membrane vesicles of *E. coli* ANN0222 transformed with the control vector pMW118 or the recombinant vector expressing the *B. pseudofirmus* OF4 *mrp*

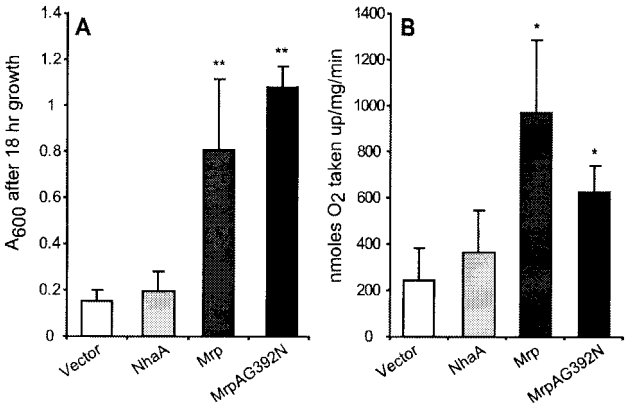

FIG. 1. Nonfermentative growth and O_2 uptake of *E. coli* ANN0222 transformants. Cells were grown on a semi-defined medium (9) containing 0.025% yeast extract, 0.1% trace salts, and 20 mM Tris–L-lactate (7). Transformants of *E. coli* ANN0222 with empty vector were compared to transformants expressing NhaA, the *B. pseudofirmus* OF4 Mrp, and a mutant of the *B. pseudofirmus* Mrp, MrpAG392N, that is deficient in Na^+ efflux. (A) A_{600} of cultures after 18 h of growth. (B) O_2 consumption by mid-log-phase cells that were transferred to a 37°C chamber, agitated, and assayed for O_2 consumption with a Clarke-type electrode. The rate of O_2 uptake is expressed in nanomoles of O_2 per milligram per minute. The results here are averages of at least four independent experiments, with error bars indicating the standard deviations from the mean. * and **, $P < 0.05$ and $P < 0.01$, respectively, by two-sample unpooled variance *t* test (StatPlot).

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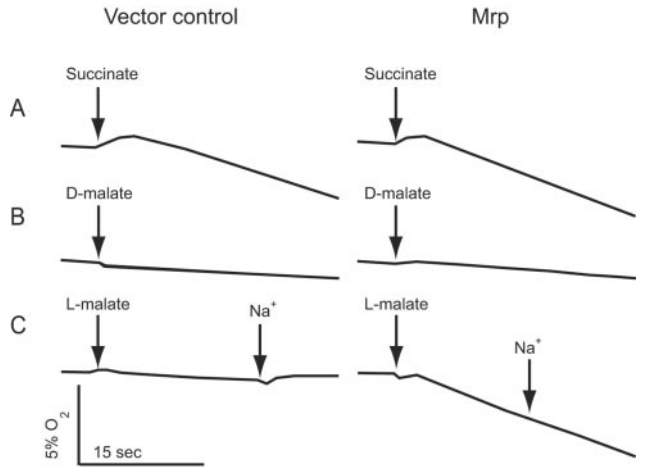

FIG. 2. Screening of electron donors for Mrp for their effect on O_2 consumption by everted *E. coli* ANN0222 vesicles. Everted membrane vesicles from control and *mrp*-expressing *E. coli* ANN0222 were assayed for O_2 consumption in response to numerous candidate electron donors, monitored with a Clark-type oxygen electrode as described elsewhere (24). The assay mix contained 300 μg of everted vesicles in 10 mM Tris-MOPS (morpholinepropanesulfonic acid)–25 mM $MgSO_4$–10% glycerol, pH 7.5. Results are shown for control and Mrp vesicles with succinate-dependent O_2 consumption as a positive control (A), D-malate-dependent O_2 consumption as a negative control (B), and L-malate-dependent O_2 consumption with the addition of 100 mM NaCl at the arrow (C).

operon (7). A positive control, succinate, supplies electrons to the terminal oxidases via the host succinate dehydrogenase. Succinate supported O_2 uptake similarly in the two vesicle preparations (Fig. 2A), while among the many test compounds, including D-malate (Fig. 2B), only L-malate significantly increased oxygen uptake in an Mrp-specific manner (Fig. 2C). Although the absolute values varied among independent experiments, the ratio of Mrp vesicles to control vesicles with succinate was 1.5/1, while the ratio with L-malate was reproducibly 4/1. Importantly, this increase was not dependent upon added Na^+ (Fig. 2C), as would be expected if it depended upon a primary Na^+ extrusion mechanism (1). Similarly, Mrp- and L-malate-dependent $\Delta\Psi$ generation was observed in a fluorescence assay of the everted vesicles (Fig. 3A and B), but Na^+ had no stimulatory effect (Fig. 3C). Na_2SO_4 was routinely used instead of NaCl since chloride ions reduce the $\Delta\Psi$ of energized *E. coli* vesicles (17). However, other sodium salts, including NaCl and $NaHCO_3$, also failed to stimulate. Moreover, no $\Delta\Psi$ generation was observed in the presence of cyanide and added quinone (Fig. 3D), as would be expected if Mrp itself is an L-malate:quinone oxidoreductase (MQO).

Mrp-dependent MQO activity of the vesicles was assayed by phenazine methosulfate-mediated bleaching of the electron acceptor 2,6-dichlorophenolinophenol (DCPIP) (4, 16). Protein was measured by the Lowry method (12). The MQO activities were 0.7 and 0.1 μmol/min/mg of protein in the *mrp*-expressing and control vesicles, respectively; the activity of succinate dehydrogenase, a positive control, was 2 μmol/min/mg of protein in both types of vesicles. The Mrp-dependent MQO activity in the *E. coli* ANN0222 vesicles was unaffected by added Na^+, and no Mrp- and L-malate-dependent $^{22}Na^+$ accumulation was observed in everted membrane vesicles of transformants of antiporter-deficient *E. coli* KNabc (Δ*nhaA* Δ*nhaB* Δ*chaA*) (18; data not shown). Although *E. coli* MQO is the product of a single gene and is a peripheral enzyme that only adheres modestly to membranes (11, 15, 16, 23), we investigated the possibility that *mrp* expression caused a large secondary increase in *E. coli mqo* expression and a corresponding increase in membrane-associated MQO. We also sought to determine whether such an increase occurred specifically in the respiration-deficient *E. coli* strain ANN0222, which has diminished capacity for respiration-dependent proton pumping and $\Delta\Psi$ generation. MQO activity was assayed in the membrane and cytosolic fractions of three *E. coli* transformants: *E. coli* ANN0222 grown on L-lactate (8) and *E. coli* KNabc and the wild-type *E. coli* strain DH5α (Gibco-BRL) grown in LBK (3). Na^+ (200 mM) was added to the growth medium of the *mrp* transformant of *E. coli* KNabc; this concentration is inhibitory for the control transformant and ensured that the *mrp* transformant was expressing an active antiporter. There were strain-specific variations in total MQO activity, but only *E. coli* ANN0222 exhibited a Mrp-dependent increase in overall MQO activity. The MQO activity of *mrp*

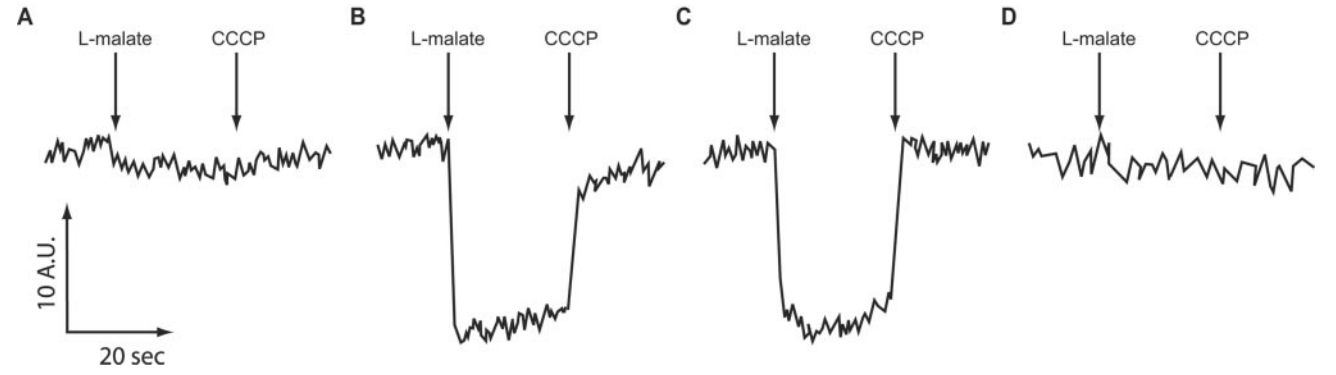

FIG. 3. Assessment of Mrp- and L-malate-dependent $\Delta\Psi$ generation in everted *E. coli* ANN0222 vesicles. Everted membrane vesicles from control and *mrp*-expressing *E. coli* ANN0222 were assayed for $\Delta\Psi$ generation in response to L-malate, monitored by quenching of oxonol VI as described elsewhere (24). The assay mix contained 200 μg of everted vesicles in 10 mM Tris-HEPES, pH 7.5. Results are shown for ANN0222/pMW118 control vesicles (A), ANN0222/Mrp vesicles (B), and ANN0222/Mrp vesicles with 10 mM Na_2SO_4 (C). For panel D, 10 mM KCN and 10 μM menadione were added 1 min before energization of the ANN0222/Mrp vesicles; the results were identical with and without 10 mM Na_2SO_4. Each trace is representative of at least three independent experiments. In each experiment, the traces were repeated two to three times. A.U., arbitrary units.

TABLE 1. MQO activity of the membrane and supernatant fractions of control and *mrp*-expressing transformants of three *E. coli* strains

Fraction	MQO activity (100 μmol/min)[a]					
	ANN0222		KNabc		DH5α	
	Vector	Mrp	Vector	Mrp	Vector	Mrp
Membranes	0.10 (12)	0.51 (15)	0.59 (18)	0.57 (17)	0.16 (6)	0.070 (6)
Cytoplasm	0.73 (88)	2.98 (85)	2.66 (82)	2.77 (83)	2.52 (94)	1.15 (94)
Membranes + cytoplasm	0.83	3.49	3.25	3.34	2.68	1.22

[a] Control (Vector) or *mrp* (Mrp) transformants of the indicated *E. coli* strains were fractionated after French pressure cell treatment for vesicle preparation, and both the membrane and supernatant fractions were assayed for MQO activity and protein. DCPIP bleaching was monitored via A_{600} in assay mixtures of 1 ml containing 100 to 200 μg of vesicles suspended in 10 mM bis-Trispropane-sulfate–5 mM $MgSO_4$, pH 7.5, and assayed as described previously (4, 16). Protein was measured by the Lowry method (12). The values in the table are total units of MQO in the indicated fraction(s). The values were highly reproducible among duplicate determinations in independent experiments. The values in parentheses are the percentage of each fraction of the total units for that strain.

transformant membranes was about four times higher than that of the control preparation (Table 1). The membrane fractions from the different strains contained 10 to 20% of the total MQO activity, consistent with the literature values (23).

The inference from the data in Table 1 is that the Mrp-dependent stimulation of *E. coli* ANN0222 growth on L-lactate results from an indirect effect of expression of the *mrp* operon that is not related to the Na^+/H^+ antiport capacity of Mrp. We tested the capacity of a mutant *mrp* that lacks Na^+/H^+ activity to enhance nonfermentative growth of the respiratory chain mutant. An MrpA-G392N mutation was introduced into the wild-type *mrp* operon cloned in pMW118 by the method of Horton (6). Consistent with loss of Na^+/H^+ antiport activity by a mutation in the same position of another alkaliphile, *mrpA* (3), this mutant *mrp* did not confer Na^+ resistance in Na^+-sensitive *E. coli* KNabc but the same mutant plasmid still complemented the growth and oxygen uptake phenotypes of *E. coli* ANN0222 (Fig. 1). The data negate the hypothesis that Mrp-dependent stimulation of nonfermentative growth and oxygen uptake of *E. coli* ANN0222 indicates a primary energization mode for Mrp. Rather, endogenous MQO can partially complement the *E. coli* ANN0222 phenotype when its expression increases as a secondary result of some effect of *mrp*. We do not know precisely how expression of either a functional or nonfunctional *mrp* operon leads to increased MQO only in the respiration-deficient strain. Perhaps expression of these heterologous Mrp membrane proteins from a multicopy plasmid creates a minor ion leak in the membrane. Whereas strains with a normal respiratory chain might easily compensate via respiration-dependent proton extrusion, the resulting depolarization or altered cytoplasmic ion complement of *E. coli* ANN0222 may lead to *mqo* induction.

The similarity between several Mrp proteins and membrane-embedded subunits of ion-coupled NADH dehydrogenases probably reflects common functions in the cation conducting pathway in the these respiratory chain complexes and in a secondary Mrp antiporter system (2, 14, 22). The basis for the requirement for an unusual number of gene products for this antiporter system is yet to be resolved. Given the efficacy of Mrp in pH homeostasis in alkaliphiles, it will be of interest to explore whether multiple Mrp proteins confer added stability or kinetic competence on this secondary Na^+/H^+ antiporter.

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