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Identification of an Na^+-Dependent Malonate Transporter of *Malonomonas rubra* and Its Dependence on Two Separate Genes

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Two membrane proteins encoded by the malonate fermentation gene cluster of *Malonomonas rubra*, MadL and MadM, have been synthesized in *Escherichia coli*. MadL and MadM were shown to function together as a malonate transport system, whereas each protein alone was unable to catalyze malonate transport. Malonate transport by MadLM is Na^+ dependent, and imposition of a ΔpNa^+ markedly enhanced the rate of malonate uptake. The kinetics of malonate uptake into *E. coli* BL21(DE3) cells synthesizing MadLM at different pH values indicated that $Hmalonate^-$ is the transported malonate species. The stimulation of malonate uptake by Na^+ ions showed Michaelis-Menten kinetics, and a K_m for Na^+ of 1.2 mM was determined. These results suggest that MadLM is an electroneutral $Na^+/Hmalonate^-$ symporter and that it is dependent on two separate genes.

Malonomonas rubra, a strictly anaerobic, gram-negative bacterium, receives its entire energy for growth from the decarboxylation of malonate to acetate and CO_2 (5). As the free energy of malonate decarboxylation ($\Delta G^{o\prime} = -17.4$ kJ/mol) is not sufficient to support the synthesis of ATP via substrate level phosphorylation, ATP synthesis must be coupled to an energy-rich ion gradient over the membrane. The decarboxylation of malonate in *M. rubra* was shown to be mediated by a distinct set of soluble and membrane-bound enzymes. The overall reaction was specifically activated by Na^+ ions and was therefore assumed to involve Na^+ ion pumping across the membrane (10, 11). Recently, the genes encoding the different proteins involved in malonate decarboxylation have been cloned and sequenced. These are located within a cluster of 14 genes. The function of most of the derived proteins could be deduced from sequence comparisons and could be related to specific steps in the malonate fermentation (1). One of the genes, *madB*, encodes a highly hydrophobic protein with significant sequence homologies to the membrane-bound decarboxylase subunit of other Na^+-pumping decarboxylases, e.g., the oxaloacetate decarboxylase from *Klebsiella pneumoniae* (1, 7). Consequently, it was concluded that the malonate decarboxylase from *M. rubra* is also a member of the Na^+ transport decarboxylase family of enzymes. The $\Delta\mu Na^+$ (electrochemical sodium ion gradient) generated must be used for the synthesis of ATP via an F_1F_0 ATPase. Part of the driving force could be consumed for the uptake of the growth substrate, malonate, into the cells. To address this possibility, we considered the gene for the putative malonate transporter to be located within the malonate fermentation gene cluster. Hydropathy analyses of the deduced gene products of this cluster indicated that along with MadB, MadL and MadM are integral membrane proteins (1). In this work, we concentrated on the *madL* and *madM* genes and their functional expression in *Escherichia coli*. The results indicate that the malonate carrier of *M. rubra* is dependent on both genes and catalyzes the ΔpNa^+-driven transport of $Hmalonate^-$ over the membrane.

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MATERIALS AND METHODS

Materials. *E. coli* DH5α was used for general cloning purposes, and *E. coli* BL21(DE3) was used for expression of the *madL* and *madM* genes. pET-24a(+) (Novagen) and pACYC184 (New England Biolabs) were used as cloning and expression vectors.

General procedures. Luria Bertani (LB) broth and LB agar were used for routine bacterial growth (19). Kanamycin was added at 50 μg ml^{-1}, and chloramphenicol was added at 40 μg ml^{-1}. Recombinant DNA procedures were performed as described previously (19). DNA fragments from agarose gels were isolated with QIAEX (Qiagen). Cloned PCR fragments were sequenced according to the dideoxy nucleotide chain termination method (20) by using an Amplitaq DNA sequencing kit and the model 310 Genetic Analyzer from Applied Biosystems.

Construction of the expression vectors. The following mixture (50 μl) was used for PCR: 20 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.5 mg of bovine serum albumin per ml, 2 mM concentrations of each deoxynucleoside triphosphate, 2.6 μM site-specific primers, and 100 ng of genomic DNA from *M. rubra* as template. Two units of native *Pfu* polymerase (Stratagene) was added. After an initial denaturation step at 94°C for 3 min, 35 cycles were carried out for 30 s at 94°C, 30 s at 60°C, and 3 min at 75°C. The forward primer madLM-for (5′-GGA GTC TCA TAT GGT CAT CTA TGG GGT A-3′) is identical to bases 9656 to 9683 of the *M. rubra mad* gene cluster (GenBank database accession number U89780), introducing a cleavage site for the restriction endonuclease *Nde*I (altered bases are underlined). The reverse primer madLM-rev2 (5′-ATT AGA AGC TTT CCG TAA ATG CCC TTC ATA A-3′) is complementary to the bases 10825 to 10855. The primer introduces a cleavage site for the restriction endonuclease *Hin*dIII (altered bases are underlined) and deletes the original stop codon of *madM*. The PCR product was restricted with *Nde*I/*Hin*dIII and ligated into *Nde*I/*Hin*dIII-restricted pET-24a(+), yielding pET-LM. Plasmid pET-LM carries a gene encoding, in addition to MadL, MadM comprising a C-terminal tag of six consecutive histidine residues (His tag). Subsequently, pET-LM was restricted with *Hin*dIII and *Bse*RI, treated with T4 DNA polymerase, and religated to obtain pET-L. For construction of pET-M, pET-LM was restricted with *Nde*I and *Nhe*I and the recessed 3′ termini were filled in with Klenow polymerase and religated. The plasmids pET-L and pET-M were restricted with *Bpu*1102I, the overhanging ends were filled in a Klenow reaction, and the resulting fragments were subsequently digested with *Bgl*II. The *madL*- or *madM*-bearing fragments were ligated into *Bam*HI- and *Nru*I-restricted pACYC184 to obtain pACYC-L and pACYC-M, respectively. The DNA sequence of the inserts carrying the *madL* and *madM* genes and the fusion sites were confirmed by sequencing.

Expression of MadL and MadM. *E. coli* BL21(DE3) cells harboring the indicated plasmid constructs were grown aerobically on LB medium (1 liter) supplemented with the appropriate antibiotics at 37°C with shaking at 180 rpm. At an optical density at 600 nm of 0.8 to 1.2, IPTG (isopropyl-β-D-thiogalactopyranoside) (final concentration, 0.2 mM) was added and the culture was grown for another 1.5 h at 37°C with shaking at 180 rpm. Cells were washed at least three times with Na^+-poor 50 mM potassium phosphate, pH 7.0, unless indicated

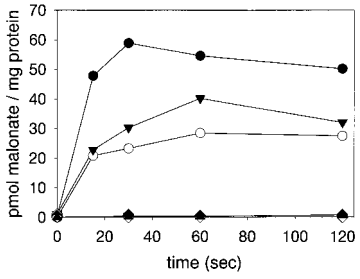

FIG. 1. Malonate uptake into *E. coli* cells expressing *madL* and/or *madM*. *E. coli* BL21(DE3) cells transformed with pET-LM (expressing *madL* and *madM* in *cis*) (●) or pACYC-M and pET-L (*trans*) (○) or pACYC-L and pET-M (*trans*) (▼) were compared. In controls, *E. coli* BL21(DE3) was transformed with pET-24a(+) (vector control) (▲), pET-L (expression of *madL*) (◆), pET-M (*madM*) (◇), pACYC-L (*madL*) (▼), or pACYC-M (*madM*) (▽). Cells in 50 mM potassium phosphate buffer, pH 7.0, containing 10 mM NaCl (99 μl) were incubated for 1 min at room temperature, prior to the addition of 1 μl of [2-^{14}C]malonate (1.8 mM; specific activity, 113 cpm/pmol). After the times indicated, samples were treated as described in Materials and Methods.

otherwise, until a Na^+ concentration of about 25 μM was obtained. Cells were resuspended in the same buffer at a concentration of about 10 to 15 mg of protein/ml and stored on ice until use.

Malonate transport assays. Transport measurements were performed at room temperature. Malonate transport was initiated by addition of 1 μl of [2-^{14}C]malonic acid (1.8 mM, 56 mCi/mmol; ICN) to 99 μl of cells in 50 mM potassium-phosphate, pH 7.0, containing 10 mM NaCl. The transport reaction was stopped after various times by addition of 900 μl of ice-cold 0.1 M LiCl to the sample and rapid filtration through 0.45-μm-pore-size cellulose nitrate filters (Sartorius). The filters were washed once with 1 ml of ice-cold 0.1 M LiCl and placed into scintillation vials. After addition of 4 ml of scintillation fluid (Irga-Safe$^+$; Canberra Packard), the entrapped [2-^{14}C]malonate was determined with a liquid scintillation counter. Experimental values were corrected for zero-time controls by simultaneous addition of [2-^{14}C]malonic acid (1 μl, 1.8 mM) and 900 μl of ice-cold 0.1 M LiCl to 99 μl of cells, followed by rapid filtration and washing of the sample as described above.

(i) Effect of alkali ions on the rate of malonate transport. To investigate the effect of Na^+ and Li^+ ions on malonate transport, *E. coli* BL21(DE3)/pET-LM cells (97 μl) suspended in 50 mM potassium phosphate buffer, pH 7.5, were preincubated with 1 μl of 0.5 M NaCl or LiCl for 1 min at room temperature prior to the addition of 2 μl of 2.9 mM [2-^{14}C]malonate (34,730 cpm/nmol of specific activity) to start the transport process. ΔpNa^+-driven malonate uptake was initiated by addition of 3 μl of 1.67 M NaCl, containing 1.93 mM [2-^{14}C]malonate (34,730 cpm/nmol), to 97 μl of Na^+-free *E. coli* BL21(DE3)/pET-LM cells. After given times, the reaction was terminated by dilution and filtration as described above.

(ii) Determination of the transported malonate species. To determine the kinetic constants (K_m, V_{max}) of MadLM for malonate, transport experiments were performed with *E. coli* BL21(DE3)/pET-LM cells (suspended in 50 mM potassium phosphate, Na^+ free) at three different pH values (6.5, 7.0, and 7.5). Transport was essentially performed as described above except that the final NaCl concentration used in this assay was 50 mM. Radioactively labeled malonate was added to final concentrations ranging from 4 to 100 μM. The initial rate values were determined from 30-s measurements. The data sets were analyzed by fitting to the Michaelis-Menten or Hill equation (4) by using the computer program Sigma Plot (Jandel Scientific, SSPS). The apparent K_ms (K_m^{app}s) for the individual malonate species (H_2malonate, Hmalonate$^-$, and malonate^{2-}) were derived by using the Henderson-Hasselbach equation.

(iii) Determination of the K_m for Na^+. To determine the Na^+ kinetics of Na^+/malonate uptake, transport assays were performed with Na^+-free washed *E. coli* BL21(DE3)/pET-LM cells at pH 6.5. The transport assay was started by simultaneous addition of NaCl (0.05 to 10 mM final concentration) and [2-^{14}C]malonic acid (final concentration, 18 μM). Mixtures of NaCl and KCl were used to provide equal salt concentrations (10 mM chloride) in the separate experiments. Data sets were analyzed by fitting to the Michaelis-Menten or Hill equation as described above.

Other methods. Protein concentration was measured by the method of Bradford (3) by using bovine serum albumin as a standard. Na^+ concentrations were determined with an atomic absorption-flame emission spectrophotometer (Shimadzu, type AA-646).

RESULTS

Cloning and expression of the *madL* and *madM* genes. The *madL* and *madM* genes were amplified from genomic *M. rubra* DNA. The primers used in this PCR procedure were designed to introduce restriction sites (*Nde*I and *Hin*dIII) flanking the genes and to delete the stop codon of the *madM* gene in the resulting amplification product. After restriction with *Nde*I and *Hin*dIII, the PCR fragment was cloned into pET24a(+), yielding pET-LM. This construct encodes (besides MadL) a MadM protein including a 6× poly-histidine extension (His tag) at its C terminus. Deletion of the *madL* or *madM* gene by restriction and religation of pET-LM resulted in the creation of pET-L and pET-M, respectively. Furthermore, pET-L and pET-M served as donors for the *madL* and *madM* genes present in the pACYC184 derivatives, pACYC-L and pACYC-M, respectively. These plasmids were used to investigate the function of the *madL* and *madM* gene products in *E. coli* as host. *E. coli* BL21(DE3) cells expressing *madL* and/or *madM* were sensitive to induction by IPTG (0.2 mM), which led to severe growth inhibition. Due to the limited synthesis of the *madL* and *madM* gene products, these proteins could not be detected by sodium dodecyl sulfate-gel electrophoresis analysis. Therefore, expression was monitored by uptake of [2-^{14}C]malonate into cells harvested about 90 min after induction with IPTG.

Concomitant expression of *madL* and *madM* is required to evoke transport of [2-^{14}C]malonate in *E. coli*. Since *E. coli* can neither grow nor metabolize malonate (12, 13), it provides an excellent system for studying malonate uptake transporters. Uptake of [2-^{14}C]malonate could readily be detected in *E. coli* BL21(DE3)/pET-LM, whereas cells harboring the vector pET24a(+) or plasmids containing only one of the genes (pET-L, pET-M, pACYC-L, or pACYC-M) exhibited no malonate transport activity (Fig. 1). The expression of both *madL* and *madM* in *trans* from pET-L and pET-M in combination with the pACYC184 derivatives demonstrated that the presence of both polypeptides is imperative for malonate accumulation in the cells. Cells expressing *madL* and *madM* in *trans* showed about 40 to 50% of the malonate uptake activity of cells expressing both genes in *cis* (i.e., from plasmid pET-LM). The results clearly show the necessity for the presence of both MadL and MadM in order to render malonate uptake activity in *E. coli*.

Reversibility of the MadLM malonate uptake system. We obtained preliminary evidence for MadLM functioning as a secondary uptake system by the release of previously accumulated radioactively labeled malonate from *E. coli* BL21(DE3)/pET-LM cells upon addition of a 1,000-fold excess of unlabeled malonate (Fig. 2). In a period of 5 min, almost 90% of the previously accumulated [2-^{14}C]malonate was released from

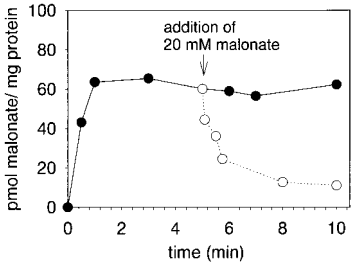

FIG. 2. Release of intracellular [2-^{14}C]malonate from *E. coli* BL21(DE3)/pET-LM by addition of 20 mM external malonate. *E. coli* BL21(DE3)/pET-LM cells suspended in 50 mM potassium phosphate, pH 7.0, containing 10 mM NaCl (99 μl) were preincubated for 1 min at room temperature. Subsequently, the transport assay was initiated by addition of 1 μl of [2-^{14}C]malonate (1.8 mM; specific activity, 113 cpm/pmol). After 5 min, 100 μl of 40 mM potassium malonate (pH 7.0) was added (○). In the control (●), 100 μl of 50 mM potassium phosphate buffer (pH 7.0) was added. After the times indicated, samples were treated as described in Materials and Methods.

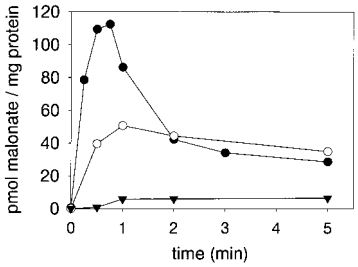

FIG. 3. Na^+ dependency of malonate transport in *E. coli* BL21(DE3)/pET-LM. Transport was initiated by addition of 2 μl of 2.9 mM [2-^{14}C]malonate (34,730 cpm/nmol) to 98 μl of cells suspended in 50 mM potassium phosphate buffer, pH 7.5, without NaCl addition (<25 μM Na^+) (▼) or with 50 mM NaCl (○). ΔpNa^+-driven malonate uptake was initiated by addition of 3 μl of 1.67 M NaCl, containing 1.93 mM [2-^{14}C]malonate (34,730 cpm/nmol), to 97 μl of Na^+-free cells (●). After the times indicated, samples were treated as described in Materials and Methods.

the cells. This type of induced release of radioactive material is quite typical for secondary active transport systems, as has been shown, e.g., for the anaerobic dicarboxylate uptake systems in *E. coli* (8). Moreover, this induced release was not observed with a primary active transport system, the aerobic dicarboxylate system of *E. coli* (8, 14). In the case of the MadLM system, efflux of radioactive malonate could not be provoked with oxalate, succinate, fumarate, and malate, the latter two being alternative growth substrates for *M. rubra* (6), indicating a narrow substrate specificity of the MadLM transport system (not shown).

Driving forces of malonate transport and ion specificity. All the transport experiments described above were performed in the presence of NaCl. To obtain information about the ionic specificity of the MadLM transport system, the Na^+ content of the cells was reduced as far as possible (<25 μM) by washing with potassium phosphate buffer. The resulting *E. coli* BL21 (DE3)/pET-LM cells with less than 25 μM Na^+ were unable to accumulate added [2-^{14}C]malonate (Fig. 3). In comparison, the same cells preincubated with 50 mM NaCl (20 min) before the addition of radioactively labeled malonate showed substantial malonate transport activity (about a 13-fold stimulation of the initial rate). These results clearly indicate that malonate transport by MadLM is Na^+ dependent. Preincubation of Na^+-poor cells with LiCl (50 mM final concentration) resulted in a stimulation of about 3.4-fold, in comparison to cells without added salts (not shown). The stimulation of malonate uptake by Li^+ ions is not surprising, since most of the Na^+-dependent transport systems can use Li^+ as an alternative coupling ion (7, 16). The highest malonate transport rate was obtained by simultaneous addition of NaCl (50 mM final concentration) and [2-^{14}C]malonate to the Na^+-free cells (imposition of a ΔpNa^+). The initial malonate uptake rate in this experiment is about 53-fold in comparison to the uptake rate of Na^+-poor washed cells and about fourfold higher than the uptake rate in cells preincubated with the same amount of NaCl. The accumulated [2-^{14}C]malonate rapidly effluxes from the cells, suggesting a rapid breakdown of the applied Na^+ gradient. The level of radioactivity remaining in the cells reached approximately the same level as observed with Na^+-equilibrated cells. The results give preliminary indication of MadLM functioning as a ΔpNa^+-driven, secondary active malonate transport system.

Affinity of MadLM for malonate. The K_ms of MadLM for malonate were determined by ΔpNa^+-driven malonate transport at three different pH values (6.5, 7.0, and 7.5). From the data obtained, K_m^{app}s of the different malonate species were calculated. As is shown in Table 1, the K_m^{app}s for the onefold-protonated malonate species ($Hmalonate^-$) remained nearly constant upon variation of the pH, whereas the K_m^{app}s for malonic acid (H_2malonate) and the unprotonated malonate ($malonate^{2-}$) changed by factors of 15 and 6.4, respectively, over the pH range tested. Assuming the variation of the pH in the range tested does not significantly affect the malonate binding site(s) of the malonate carrier, we conclude from these results that $Hmalonate^-$ is the actual substrate transported by MadLM.

Affinity of MadLM for Na^+ ions. The experiments described provided evidence for a Na^+-dependent malonate uptake system. To determine the affinity constant of MadLM for Na^+ ions, we performed uptake studies with Na^+-free washed *E. coli*(DE3)/pET-LM cells. To provide a driving force for the malonate uptake, [2-^{14}C]malonate (18 μM final concentration) and NaCl (0.05 to 10 mM final concentration) were added simultaneously to the cells. The initial transport rates were analyzed by fitting to the Hill equation (4). The calculated $K_{0.5}$ value for Na^+ at pH 6.5 was 1.19 mM, and the V_{max} value was 4 nmol/min/mg of protein. The calculated Hill coefficient was 0.96, pointing towards a noncooperative mechanism of Na^+ transport.

DISCUSSION

In this work, we demonstrate that the malonate carrier from *M. rubra* is encoded by two genes, *madL* and *madM*. Expression of both genes is imperative to render an active malonate transport system. Expression of either the *madL* or the *madM* gene in *E. coli* did not confer malonate transport activity on this host. To demonstrate that the lack of malonate uptake activity was not due to the lack of expression of *madL* or *madM*, we expressed both genes in *trans*. In this experiment, the expression of a functional malonate carrier clearly demonstrated that both genes are properly transcribed and translated (Fig. 1). Based on hydropathy plot analysis, four and seven putative transmembrane regions were predicted for MadL and MadM, respectively (1). Most secondary active carriers of bacterial origin are predicted or have been shown to consist of 10 to 12 transmembrane α helices, connected by mainly short, hydrophilic loops. The proteins usually are divided into two halves by a large hydrophilic (cytoplasmic) domain (6 + 6 symmetric arrangement) (15). Notably, if the MadLM subunit structure were heterodimeric, the transport system would theoretically contain 11 transmembrane helices in a 4 + 7 asymmetric arrangement. It is obvious from the data presented in this report that MadLM functions as a Na^+-dependent malonate transporter. More difficult to interpret is the actual driv-

TABLE 1. Kinetic analysis of malonate uptake[a]

pH	K_m^{app} (μM)				V_{max}
	H_2malonate	$Hmalonate^-$	$malonate^{2-}$	Malonate total	
6.5	0.0003	**1.39**	9.81	11.2	1.52
7.0	0.00007	**1.09**	24.41	25.5	1.01
7.5	0.00002	**0.88**	62.62	63.5	0.95

[a] Determination of the uptake of [2-^{14}C]malonate was performed with *E. coli* BL21(DE3)/pET-LM at the pH values indicated and under conditions where a ΔpNa^+ was imposed. Initial values were obtained from 30-s measurements. The concentration of the respective malonate species was calculated by using the Henderson-Hasselbach equation. The K_m^{app}s for $Hmalonate^-$ are given in bold type. The pK_as for malonate are 2.83 and 5.69. Data sets were analyzed by using Lineweaver-Burk plots. V_{max} is given in nanomoles of malonate per minute per milligram of protein.

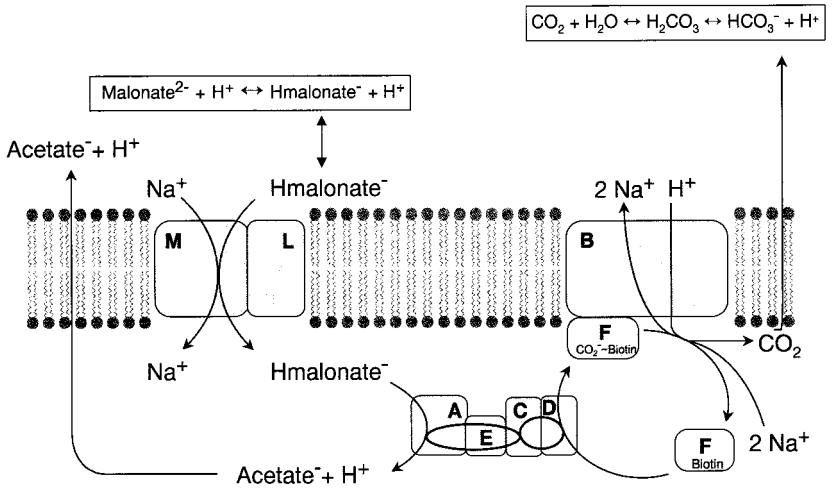

FIG. 4. Model for the Na^+ cycle during malonate fermentation in *M. rubra*. The malonate transporter (MadLM) and the intrinsic membrane part of the malonate decarboxylase (MadB) are depicted as boxes in the membrane. The biotin-containing subunit MadF, drawn at the cytoplasmic side of the membrane, functions as a CO_2 shuttle between MadB and the soluble parts of the malonate decarboxylase (MadACDE). The direction of the H^+ and Na^+ fluxes during malonate fermentation is indicated with arrows. Acetate is postulated to leave the cell by passive diffusion or via a specific carrier. Further details are explained in the text.

ing force(s) for malonate uptake into the cell. Preincubation of *E. coli* BL21(DE3)/pET-LM cells with 50 mM Na^+ (no ΔpNa^+ present) leads to accumulation of [2-^{14}C]malonate inside these cells. More significant, however, is the accumulation of the labeled malonate in the presence of a ΔpNa^+ (Fig. 3). The accumulated [2-^{14}C]malonate rapidly effluxes from the cell, probably caused by dissipation of the ΔpNa^+ by action of, e.g., Na^+/H^+ antiporters. This experiment furthermore demonstrated the reversibility of malonate transport by MadLM (which is further confirmed by the results of the exchange experiment shown in Fig. 2). Hence, a directed transport of malonate by action of a primary active transport system could be excluded. The identification of other driving forces, like $\Delta\Psi$ (electrical potential) and ΔpH, proved to be more difficult than anticipated. We successfully performed [^{14}C]proline uptake experiments with *E. coli* BL21(DE3)/pET-LM cells (in the presence of 10 mM Na^+ but no ΔpNa^+ imposed), and uptake of radioactively labeled proline could be forestalled by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (a protonophor) (data not shown). These control experiments proved the presence of a proton-motive force across the cell membrane under the conditions tested. Analogous uptake experiments in *E. coli* BL21(DE3)/pET-LM using [2-^{14}C]malonate showed that the initial uptake rate was almost unaffected by CCCP (100 μM), but the final accumulation of the substrate was about 50 to 70% that of the control without CCCP (data not shown). Moreover, investigation of [2-^{14}C]malonate uptake with EDTA-treated cells (9) in the presence or absence of valinomycin (disrupts $\Delta\Psi$) led to similar results (not shown). Unfortunately, the interpretation of the results from these experiments was too difficult to allow a final conclusion. On the other hand, the results obtained from the kinetics of malonate and Na^+ uptake experiments were essentially clear. We could determine the onefold-protonated species of malonate ($Hmalonate^-$) as the probable substrate for MadLM (Table 1). Moreover, we could demonstrate that the Na^+-dependent malonate uptake by MadLM followed Michaelis-Menten kinetics, i.e., a Hill coefficient of about 1 was found. Assuming that the presence of more than one binding site for Na^+ on the MadLM system would evoke cooperative kinetics (i.e., a Hill coefficient of >1), we tend to believe that the actual stoichiometry between Na^+ and $Hmalonate^-$ is one. This would then indicate an electroneutral $Na^+/Hmalonate^-$ symport by MadLM, and the sole driving force would be the ΔpNa^+ (and the $\Delta\mu Hmalonate^-$). To resolve this matter, we intend to purify the MadLM complex and reconstitute it into liposomes. Elucidation of the driving force(s) will then be performed with the help of artificial ion gradients, as was successfully demonstrated in the case of the Na^+-dependent citrate carrier from *K. pneumoniae* (17, 18). Because *M. rubra* growing on malonate lives at an energetical subsistence level (2 g of dry cell matter formed from 1 mol of malonate), it was surprising to find that this organism uses part of the $\Delta\mu Na^+$, created by conversion of the decarboxylation energy (malonate→acetate + CO_2), for malonate uptake. Another possibility for malonate uptake would have been malonate/acetate antiport. Since acetate is the end product of the malonate fermentation pathway, its outwardly directed gradient could have been an excellent driving force for the uptake of malonate. Experiments were performed to address this possibility, but involvement of acetate as an antiport substrate could not be shown. Acetate is therefore more likely to leave the cell either by passive diffusion or via a specific export carrier. In analogy to the Na^+ cycle operating in *K. pneumoniae* during citrate fermentation (2, 7), we present a model for Na^+ cycling during malonate fermentation in *M. rubra* (Fig. 4). Inside the cell, malonate is decarboxylated to acetate by the malonate decarboxylase (encoded by the *madABCDEF* genes [1]), coupled to the extrusion of Na^+ ions. Based on the mechanism of the well-studied oxaloacetate decarboxylase from *K. pneumoniae* (6), we presume that two Na^+ ions are extruded per decarboxylation reaction with a concomitant consumption of one H^+ originating from the extracellular environment. Another proton enters the cell by the uptake of $Hmalonate^-$. On the other hand, protons are extruded due to the efflux of acetic acid, and by the diffusion of CO_2 to the external medium and its subsequent conversion into HCO_3^- and H^+. As the first pK_a of malonic acid (2.83) is 3.5 units less than that of the carbonic acid produced (6.35), the medium turns slightly alkaline (about 0.6 pH units) during anaerobic growth of *M. rubra* on malonate under conditions where a 2×10^4 Pa overpressure of N_2/CO_2 is applied (not shown). The difference between the second pK_a of malonic acid (5.69) and that of the second end product, acetic acid (4.76), cannot compensate for the observed alkanization. The

uptake of malonate by the MadLM symporter proceeds electroneutrally at the expense of 1 Na^+ ion. This leaves the cell with one Na^+ ion (including charge) to perform other vital processes like ATP synthesis by action of an F_1F_0-ATP synthase. The use of an electroneutral uptake system for malonate in *M. rubra* in fact prevents the dissipation of the $\Delta\Psi$ created by the decarboxylase and reserves this driving force for the synthesis of ATP.

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