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Characterization of Fumarate Transport in *Helicobacter pylori*

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Abstract. The fumarate transport system of the bacterium *Helicobacter pylori* was investigated employing radioactive tracer analysis. The transport of fumarate at micromolar concentrations was saturable with a K_M of 220 ± 21 μM and V_{max} of 54 ± 2 nmole/min/mg protein at 20°C, depended on temperature between 4 and 40°C, and was susceptible to inhibitors, suggesting the presence of one or more fumarate carriers. The release of fumarate from cells was also saturable with a K_M of 464 ± 71 μM and V_{max} of 22 ± 2 nmol/min/mg protein at 20°C. The rates of fumarate influx at millomolar concentrations increased linearly with permeant concentration, and depended on the age of the cells. The transport system was specific for dicarboxylic acids suggesting that fumarate is taken up via dicarboxylate transporters. Succinate and fumarate appeared to form an antiport system. The properties of fumarate transport were elucidated by investigating the effects of amino acids, monovalent cations, pH and potential inhibitors. The results provided evidence that influx and efflux of fumarate at low concentrations from *H. pylori* cells was a carrier-mediated secondary transport with the driving force supplied by the chemical gradient of the anion. The anaerobic C_4-dicarboxylate transport protein identified in the genome of the bacterium appeared to be a good candidate for the fumarate transporter.

Key words: Fumarate — Dicarboxylic acid — Transport — Radiotracer analyses — *Helicobacter pylori*

Introduction

The Gram-negative, microaerophilic, vibrioid bacterium *Helicobacter pylori* has a unique ecological niche in the human upper gastrointestinal tract [30]. The bacterium has been established as the major aetiological agent of acute chronic gastritis [40], associated with peptic ulcers [6, 18], and more recently linked to the development of gastric cancer [25, 49].

In its natural habitat on the lining of the stomach gastric epithelium, *H. pylori* is exposed to relatively high concentrations of host breakdown products including amino acids and dicarboxylic acids [4]. The bacterium may obtain its nutrients from blood supplying the epithelial cells and intercellular junctions [20], and histology studies have shown the region of the gastric mucosa colonised by *H. pylori* to be relatively free of other microbial flora [44]. Several amino acids including asparagine and aspartate are deaminated at fast rates by the bacterium and fumarate is a main product of the catabolism of these two amino acids [34]. *H. pylori* metabolizes fumarate [33, 39]; and the presence of intermediary fermentative metabolism has been established [8, 34, 35, 36, 37]. Conversion of pyruvate to acetate and formate provided evidence for the existence of mixed-acid fermentation pathway in *H. pylori;* and the accumulation of lactate from pyruvate showed the presence of fermentative lactate dehydrogenase activity in the microorganism [34]. Fumarate at high concentrations is an inhibitor of *H. pylori* fumarate reductase, a key component of fumarate respiration in the bacterium [39]. Since the influx of many metabolic fuels and precursors and efflux of catabolic products is controlled by specific transport systems in the cell membranes of bacteria, a complete understanding of fumarate metabolism in *H. pylori* required the investigation of the mechanisms that regulate the transport of this metabolite.

Inducible C_4-dicarboxylate transport systems have been observed in *Azotobacter vinelandii* [42], *Bacillus subtilis* [16], *Bradyrhizobium japonicum* [24], *Escherichia coli* [13, 27], *Enterobacter aerogenes* [24], *Pseudomonas putida* [9], *Rhizobium leguminosarum* [14, 17],

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Rhizobium japonicum [32], and *Salmonella typhimurium* [26].

In eukaryotes two separate mitochondrial transport systems for dicarboxylic acids catalize an electroneutral exchange of substrate anions: the dicarboxylate carrier and the α-ketoglutarate (2-oxoglutarate) carrier. The first type of carrier transports dicarboxylates but not α-ketoglutarate, and the second type accepts α-ketoglutarate and some dicarboxylates [3].

Radioactive tracer analysis techniques using [2,3-^{14}C] fumarate were employed to determine the kinetic parameters, temperature dependence, substrate specificity, and the effects of cations and inhibitors on fumarate transport in *H. pylori;* as well as the metabolic fate of the label incorporated into the cellular mass.

Materials and Methods

Materials

Amino acids, 5-(N,N-Dimethyl)-amiloride hydrochloride, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), carboxylic acids, *p*-chloromercuribenzoic acid, 2,4-dinitrophenol (DNP), [2,3-^{14}C]fumaric acid (10.2 mCi/mmol), fumaric acid, furosemide (5-[aminosulfonyl]-4-chloro-2-[(furanylmethyl)-amino]benzoic acid), iodoacetamide, monensin, nigericin, oligomycin, ouabain (G-strophanthin) and valinomycin were from Sigma (St. Louis, MO). [^{3}H]H_2O (100 mCi/g) was from New England Nuclear (Du Pont; North Ryde, NSW, Australia), and [U-^{14}C]taurine (115 mCi/mmol) from Amersham Life Sciences (North Ryde, NSW, Australia). All reagents were of analytical grade. Solutions were prepared in phosphate buffered saline (PBS) (in mM): 1.8 KH_2PO_4/4.8 K_2HPO_4/150 NaCl, pH 7.0, unless otherwise stated.

Bacterial Cultures and Preparation

H. pylori strain NCTC 11639 and isolate UNSW 10536/11 were grown on Blood Base Agar No. 2 (Oxoid; Basingstoke, UK) supplemented with 5 % (v/v) horse blood. UNSW 10536/11 is a low passage isolate obtained by endoscopy from a patient infected with *H. pylori*, and can be obtained from the culture collection at the University of N.S.W. Cultures were passaged every 28–32 hr and incubated in an atmosphere of 10% CO_2 in air, 95% humidity at 37°C. For transport studies, cells were harvested after 24 hr in 0.9% (w/v) NaCl, checked for purity under phase contrast microscopy, and tested for positive urease and catalase activity. Cell preparations were centrifuged at 17,000 × *g* for 8 min at 6°C. The pellet was washed twice in PBS. The cells were then resuspended in PBS for transport assays. For temperature-dependent studies, cell preparations were incubated for 20 min at the required temperature before commencement of the assay. For studies of the effects of monovalent cations on transport, cells were harvested in the corresponding chloride salt (150 mM) and washed in PBS constituted with the appropriate alkali cation.

The morphology of cultured *H. pylori* cells changes with incubation time. Cells have spiral-rod forms when they are in log phase, this shape becomes spherical with the formation of coccoids as cells grow older [7, 45]. Under the experimental conditions in which cells were grown on plates, inspection of cultures showed that after 24 hr growth more than 95% of the cells were in the spiral-rod form, and after 72 hr growth more than 95% of the cells were coccoidal.

H. pylori cells were grown in liquid cultures of semidefined media consisting of Isosensitest Broth (Oxoid; Basingstoke, UK) to which filtered (0.2 μm Minisart, Sartorius; Gröningen, Germany) solutions of bovine serum albumin and bovine liver catalase were added to final concentrations of 0.5% and 0.1%, respectively. Cells grown on plates were suspended in sterile 150 mM saline, inoculated into the media and incubated in 50 ml sterile vented tissue flasks (Corning, Cambridge, MA) in the same atmosphere employed for plate cultures.

Escherichia coli strain K12 cells were grown anaerobically on Luria plates in jars with a gas pack (Oxoid; Basingstoke, UK) at 37°C. For transport experiments, cells were harvested after 10 hr and suspensions in PBS were prepared in the same way as for *H. pylori* cells.

Measurement of Radioactivity

Samples were added to 10 ml of either Scintillant A [0.5% (w/v) 2,5-diphenyloxazole in Triton X100:toluene 1:2 (v/v)] or Scintillant B [0.5% (w/v) 2,5-diphenyloxazole in toluene]. Radioactivity was counted using a Packard 1900TR Liquid Scintillation Analyzer.

Transport Assays by Centrifugation Through Oil

In the centrifugation through oil method, 100 μl of permeant was layered onto 150 μl oil [di-n-butylphthalate:di-iso-octylphthalate 4:1 (v/v)] and 100 μl of bacterial suspension, containing approximately 3.5 × 10^8 cells/ml, was mixed with the permeant for the required time. The mixture was then centrifuged in a Microfuge E (Beckman, Gladesville, NSW, Australia) for 40 sec to pellet the cells through the oil and stop the reaction. The aqueous layer was aspirated and the surface of the oil washed three times with deionized water and then aspirated. After the oil was removed, 400 μl of 1% (v/v) Triton X100 was added to the pellet, and the mixture allowed to stand overnight. Perchloric acid [750 μl, 5% (v/v)] was then added to the mixture and incubated in an ice bath for 30 min. The suspension was centrifuged at 17,000 × *g* for 8 min at 6°C, and 1 ml of the resulting supernatant was added to Scintillant A and counted. In each experiment the total water space of the pellet was determined by the same method using ^3H_2O instead of the permeant; and the extracellular space of the pellet was measured using the impermeant [^{14}C]taurine [38]. The concentration of permeant in the extracellular space of the pellet (extracellular trapping) was the same as in the aqueous layer remaining above the oil, and in which the cells were suspended. Consistent values for the extracellular space were obtained with an average value of 70 ± 8% of the pellet. The intra- and extracellular volumes depended on the cell preparation; typical values were 2.4 and 5.7 μl, respectively; with a protein content of approximately 7.6 μg/μl.

Efflux of fumarate from cells was measured by a variation of the centrifugation through oil method. Twenty microliters of fumarate at the desired concentration were added to 180 μl of cells suspended in PBS, and the suspensions incubated for 15 min. Half of the sample was layered onto 150 μl oil and immediately spun through the oil; the *total amount of intracellular fumarate* was measured following the protocol described above. The other 100 μl of sample were centrifuged at the same time in a tube without oil. The supernatant was removed and the pellet was resuspended in 100 μl of PBS without any permeant. This suspension was layered onto oil, and after a set time from the moment in which the cells were resuspended in PBS, the sample was centrifuged through the oil; the amount of *fumarate remaining inside* the cells after the set time was determined following the protocol described above. The *fumarate released* from the cells was the difference between the total intracellular amount and the amount remaining inside the cells. In each experiment the radioactivity of the pellet, the sizes of

intra- and extracellular spaces, and the permeant concentration in the extracellular space (extracellular trapping) were measured as above.

The effects of the experimental conditions on the ability of bacterial cells to grow and proliferate was ascertained by incubating them in PBS at 37°C for different amounts of time up to 30 min. After these treatments *H. pylori* and *E. coli* cells remained fully viable when grown under microaerophilic and anaerobic conditions, respectively.

Kinetic Analyses

The kinetic constants K_M and V_{max} were determined by nonlinear regression analysis employing the program Enzyme Kinetics (Trinity Software; Campton, NH). Errors are quoted as standard deviations unless otherwise indicated.

Nuclear Magnetic Resonance Spectroscopy

Lysates suspended in PBS were placed into 5-mm tubes (Wilmad; Buena, NJ) for nuclear magnetic resonance (NMR) measurements. Free induction decays were collected using a Bruker DMX-600 NMR spectrometer, operating in the pulsed Fourier transform mode with quadrature detection. Measurements were carried out at 37°C. One-dimensional ^{1}H-NMR spectra were acquired at 600.13 MHz with presaturation of the water resonance. The instrumental parameters were: spectral width 5,000 Hz, memory size 16 K, acquisition time 1.638 sec, number of transients 288, and relaxation delay with solvent presaturation 1.4 sec. No window function was applied prior to Fourier transformation.

Effects of Carboxylic Acids and Amino Acids on Fumarate Transport

Amino acids and carboxylic acids were prepared in concentrated solutions in PBS and layered together with 100 μM of labelled fumarate onto 150 μl of oil. Cell suspensions (100 μl) were added and after 30 sec the mixtures were centrifuged through the oil at 20°C; the amount of fumarate transported into the cells was measured as described above.

Effects of Potential Inhibitors

The effects of potential inhibitors of fumarate transport were investigated by incubating 100 μl of cells with each of the inhibitors for 30 min at 37°C. From this point on two protocols were followed: in the first, 100 μM of labeled fumarate were added to the suspensions, and after 30 sec the centrifugation through oil transport assay was performed at 20°C; in the second, the cell suspensions containing the inhibitors were centrifuged, the supernatants removed, the cells resuspended in 100-μl PBS containing 100-μM labeled fumarate, and after 30 sec the assay was performed. The objective of employing two different protocols was to measure permeant transport in the presence and absence of inhibitors. Water soluble inhibitors were prepared in concentrated solutions in PBS; stock solutions of amiloride, ouabain, monensin, nigericin, oligomycin and valinomycin were prepared in absolute ethanol and then diluted in PBS so that the final concentration of ethanol in the cell suspensions was less than 1%. Control assays with the same amount of alcohol added to the cell suspensions were incubated for the same time and conditions.

Incorporation of Fumarate Carbon Atoms to the Cellular Mass

Radioactive fumarate was dissolved in 0.5 ml of water and filtered into 10 ml of *H. pylori* liquid cultures to give a radioactivity of approximately 0.1 μCi/ml. Two cultures were incubated in each experiment with 8 mM-fumarate, and the experiments were repeated five times. Duplicate 1-ml aliquots were taken from each culture at 24 hr. Cells were removed from the incubation media by centrifugation (14,000 × *g*, 3 min, 20°C). The pellet was resuspended in 1 ml of PBS and centrifuged again. After repeating the washing three times, the cells were resuspended finally in 100-μl PBS and lysed by suspending them in 470-μl Tris-EDTA (25-mM Tris, 10-mM EDTA) buffer and 30-μl 10% sodium dodecyl sulfate. Genomic DNA was prepared from bacterial lysates by removing lipids, proteins by digestion with proteinase *K*, and selective precipitation with CTAB of cell wall debris, polysaccharides and remaining proteins [1]. Fractions were collected on 1.2 μM GF/C filter paper by vacuum filtration and dried. Scintillation fluid B (10 ml) was added to the filters containing the DNA, lipids, proteins and cell debris fractions, and the radioactivity of each fraction measured. The purity of the extracted DNA was determined from the ratio of absorbances of the samples of 260 and 280 nm [2].

Statistical Analyses

Kruskal-Wallis nonparametric statistical tests of the data were performed employing the SPSS package (SPSS, Chicago, IL). Differences were considered to be significant for p values less than 0.05.

Protein Assays

Protein estimation of samples was made by the bicinchoninic acid method employing a microtitre protocol (Pierce, Rockford, IL). The values of transport rates are quoted in terms of the protein content of the samples.

Results

Influx and Efflux of Fumarate in *H. pylori* Cells

Transport of fumarate into *H. pylori* cells at 20°C was linear for 100 sec at an initial external concentration of 2 μM, it was linear for 40 sec at 1-mM permeant concentration (Fig. 1). Transport reached a maximum with time, and fumarate accumulated in cells at concentrations lower than the extracellular concentrations of the permeant (Fig. 1). Oxantel is a potent inhibitor of *H. pylori* fumarate reductase [39]; and the presence of 1-mM concentrations of the inhibitor did not affect the rates of transport at fumarate concentrations up to 500-μM (Fig. 1), establishing that there is no coupling between the influx of the dicarboxylate and its utilization in anaerobic respiration.

The reversibility of fumarate transport was established by loading the cells with labeled fumarate and observing the efflux of radioactivity as a function of time. The release of intracellular fumarate was linear for

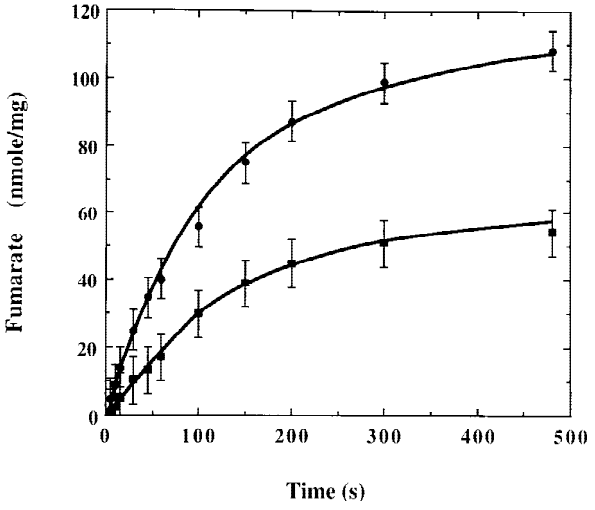

Fig. 1. Time-course of the uptake of [2,3-^{14}C]fumarate by *H. pylori* NCTC 11639 cells employing the centrifugation through oil method at 100 μM (■) and 1 mM (●) extracellular fumarate concentrations. Measurements were carried out in suspensions of cells in PBS at 20°C.

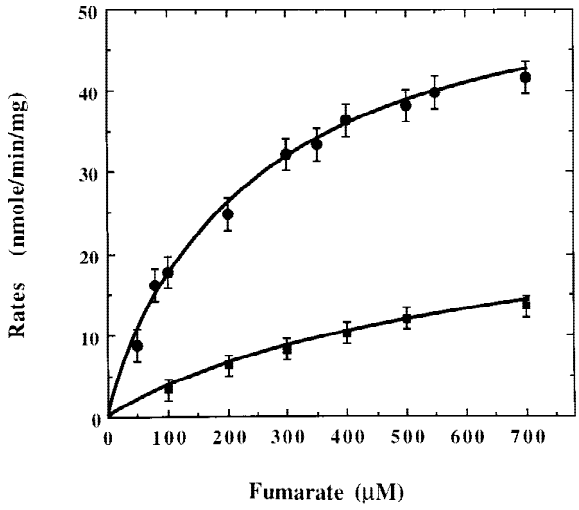

Fig. 2. Rates of fumarate (●) influx into and (■) efflux from *H. pylori* strain NCTC 11639 cells determined employing the centrifugation through oil method. Measurements were carried out in suspensions of cells in PBS at different permeant concentrations at a fixed timepoint of 30 sec and 20°C.

45 sec at a fumarate concentration of 1 mM. Isotope exchange experiments were performed by loading bacterial cells with 100-μM substrate containing radiolabeled fumarate, and measuring the rates of efflux of the radioactive anion in suspensions of unlabeled fumarate at concentrations between 0 and 2-mM external fumarate. The rates of [2,3-^{14}C] fumarate exit from the cells measured at a fixed timepoint of 30 sec and 20°C decreased with increasing extracellular concentration of unlabeled fumarate, indicating that efflux and entry of fumarate took place via the same carrier or carriers.

Kinetics of Fumarate Influx and Efflux

The kinetic parameters of fumarate transport into cells were determined over the concentration range 0 to 700 μM, at 20°C and a fixed timepoint of 30 sec. Initial rates were linear as a function of permeant concentration up to 120 μM, and the transport showed saturation (Fig. 2). Nonlinear regression analysis of the data yielded a K_M of 220 ± 21 μM and V_{max} of 54 ± 2 nmole/min/mg protein for strain NCTC 11639 ($n = 5$), and a K_M of 215 ± 21 μM and V_{max} of 52 ± 2 nmole/min/mg protein for the low passage isolate UNSW 10536/11 ($n = 3$). Within experimental error the same values were determined for the kinetic parameters of permeant influx in the presence of 1-mM oxantel, indicating that the label accumulating in the cells was fumarate.

At fumarate concentrations above 2 mM the rates of transport increased linearly as a function of permeant concentration and the transport was not saturable (*data not shown*). The rates of fumarate transport at millimolar concentrations increased with the age of the cells. At 10-mM fumarate concentration and 20°C, relative to the rates measured for cells grown for 24 hr, rates increased 30 ± 10% for cells grown for 48 hr, and 90 ± 20% for cells grown for 72 hr ($n = 4$). The morphology of bacteria in the cultures was examined with phase-contrast microscopy. Cells harvested after 24 hr incubation showed the rod-shaped bacillary form; after 48 hr incubation between 40 and 60% of the cells were in the spheroid coccoid form; and after 72 hr incubation more than 95% of the cells were coccoidal.

The kinetic parameters of fumarate efflux were determined over the intracellular concentration range 0 to 1 mM, at 20°C and a fixed timepoint of 30 sec. Cells were loaded with labeled fumarate in the presence of 1-mM oxantel to prevent conversion of fumarate to succinate. The efflux of fumarate from cells showed saturation (Fig. 2), and nonlinear regression analysis of the data yielded a K_M of 464 ± 71 μM and V_{max} of 22 ± 2 nmole/min/mg protein for strain NCTC 11639 ($n = 4$). Within experimental error the same values were determined for the kinetic parameters of permeant release in the presence of 1-mM oxantel, indicating that the label coming out of the cells was fumarate. The endogenous concentrations of fumarate were too low to be measured by ^{1}H-NMR spectroscopy in cell suspensions, suggesting an upper limit of 10–30 μM for the intracellular levels of the dicarboxylate. The data obtained for the rates indicated that the K_M for efflux would be higher if fumarate endogenous levels had been underestimated.

Temperature Dependence of Fumarate Transport

The dependence of fumarate transport on temperature [22] was measured at a fixed timepoint of 30 sec over the

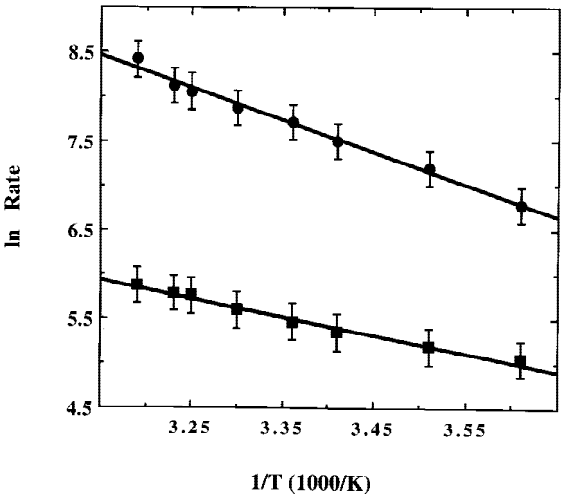

Fig. 3. Arrhenius plots of [2,3-^{14}C]fumarate transport into (●) *E. coli* K12, and (■) *H. pylori* NCTC 11639 cells. Measurements were carried out using the centrifugation through oil method in cell suspensions in PBA. Initial rates were determined at a fixed timepoint of 30 sec with labelled fumarate concentrations of 200 μM and 100 μM for *E. coli* and *H. pylori* cells, respectively

temperature range 4 to 40°C. Entry rates were dependent on temperature at permeant concentrations of 100 μM. An activation energy of 16.7 ± 2 kJ/mol for the influx of fumarate was calculated from linear Arrhenius plots ($n = 3$) (Fig. 3). For comparison, the temperature dependence of fumarate influx into *E. coli* grown under anaerobic conditions was measured at a fixed timepoint of 30 sec and a permeant concentration of 200 μM; these conditions were chosen to fit the characteristics of the anaerobic *E. coli* fumarate carrier [13]. The Arrhenius plot for *E. coli* cells was linear in the temperature range 4 to 40°C and the transport had an activation energy of 30 ± 3 kJ/mol (Fig. 3).

Specificity of the Fumarate Transport System

The specificity of the fumarate transport system was investigated by measuring the effects of the presence of other carboxylic acids at 2-mM concentrations on the transport of 100-μM fumarate at 20°C and a fixed timepoint of 30 sec. Transport of fumarate would be inhibited by carboxylic acid anions that share the carrier system with it or bind to the transporters. The results are summarized in Table 1 ($n = 4$). Statistical analysis of the data indicated that malate, maleate, malonate, oxaloacetate and succinate inhibited significantly the influx of fumarate into *H. pylori* cells; whereas glyoxylate, lactate, *cis*-aconitate and citrate enhanced fumarate transport.

Table 1. Effect of carboxylic acids on the rates of fumarate entry into strain NCTC 11639 *H. pylori* cells

Carboxylic acid anion	Activity (% of control)
Formate	117
Acetate	108
Glyoxylate	137
Lactate	140
Pyruvate	111
Malate	75
Maleate	62
Malonate	75
Oxaloacetate	67
Succinate	76
α-Ketoglutarate	131
cis-Aconitate	164
Citrate	105
Isocitrate	110

Rates were measured in cells suspended in phosphate buffered saline pH 7 and 2 mM carboxylic acid by the centrifugation through oil method at a fumarate concentration of 100 μM, 20°C and a timepoint of 30 sec. Values are quoted relative to the rates measured in solutions without any carboxylate ($n = 4$). Activities are given as the median of the measurements expressed as percentage of the control. Errors were estimated at ±10%

Effects of Succinate on the Transport of Fumarate

Succinate inhibited competitively the entry of fumarate into *H. pylori* cells. The K_i for succinate was measured at a fumarate concentration of 50 μM, 20°C, and a fixed timepoint of 30 sec. At concentrations well below the K_M the inhibition constant can be calculated from the expression

$$v_o/v = 1 + I/K_i,$$

where v_o and v are the uninhibited and inhibited rates of transport, respectively, and I is the concentration of inhibitor [12]. A K_i of 1.7 ± 0.2 mM ($n = 2$) was determined from a plot of v_o/v as a function of succinate concentration (Fig. 4).

Accumulation of succinate by *H. pylori* cells incubated with this dicarboxylic acid anion was established using ^{1}H-NMR spectroscopy. NCTC 11639 cells suspended in PBS were incubated with 10-mM succinate at 37°C for 0 and 15 min. Each suspension was layered onto oil and centrifuged. The pellet was collected, resuspended in PBS and lysed by twice-freeze-thawing. NMR spectra of the lysates showed elevated succinate concentrations in the samples incubated for 15 min, indicating that the metabolite was transported into the cells. The amount of succinate accumulated by the cells depended on the extracellular concentration of succinate during the loading period. The efflux of succinate from *H. pylori* cells has been established previously [33, 39].

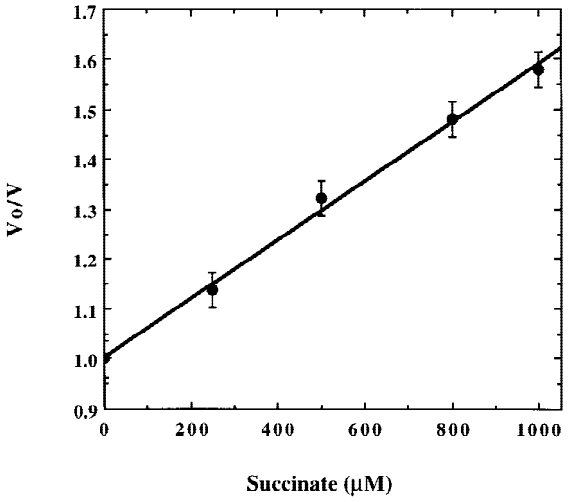

Fig. 4. Effect of succinate on the transport of 50 μM fumarate into *H. pylori* strain NCTC 11639 cells. Initial rates were determined at a fixed timepoint of 30 sec using the centrifugation through oil method at 20°C and various concentrations of succinate. v_o represents the rate of transport in the absence of succinate and v the rates in the presence of the anion.

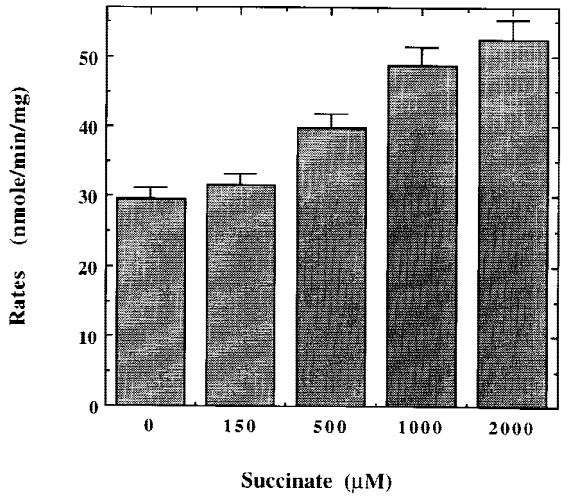

Fig. 5. Rates of transport of 100 μM fumarate into *H. pylori* strain NCTC 11639 cells loaded with succinate at different concentrations. Initial rates were determined at a fixed timepoint of 30 sec using the centrifugation through oil method at 20°C. The transport rates are shown as a function of the concentration of succinate employed to load the cells with the anion.

Cells were loaded with succinate by incubating them for 15 min at 37°C in PBS containing this metabolite. To carry out fumarate transport assays, the cells loaded with succinate were centrifuged, the supernatant removed, the pellet resuspended in PBS containing labelled fumarate, and the cells layered onto oil. After 30 sec the cell suspensions were centrifuged through the oil, and the amount of fumarate transported was measured. The effects of incubating cells with different succinate concentrations on the rates of fumarate entry are shown in Fig. 5 ($n = 3$).

Effects of Amino Acids on Fumarate Transport

H. pylori is capable of growing and proliferating employing amino acids as basic nutrients. The effects of the presence of 1-mM concentrations of these metabolites on the transport of 100-μM fumarate at 20°C and a fixed timepoint of 30 sec are shown on Table 2 ($n = 3$). Statistical analysis of the data showed that most amino acids did not affect the entry of fumarate into cells, only aliphatic (with exception of glycine) and aromatic amino acids had significant effects on fumarate transport. The effects of aliphatic amino acids on the influx of fumarate depended on their side chain length. Valine produced 36% inhibition; alanine and glycine with shorter side chains inhibited by only 25% and 2%, respectively; and leucine and isoleucine with longer side chains inhibited by 23%. Qualitatively similar results were obtained employing 10-mM amino acid concentrations. In particular, acidic and basic amino acids did not inhibit transport significantly; and no inhibition was observed with the cyclic amino acid proline or with amino acids with hydroxyl- or sulfur-containing containing side chains. On average, the strongest inhibition was measured in the presence of aromatic amino acids.

Effects of Monovalent Cations and pH on Fumarate Transport

The influence of monovalent cations on the rates of fumarate transport at 20°C and 100-μM permeant was investigated by constituting the phosphate buffer in PBS with the appropriate cation and substituting NaCl with LiCl, KCl, RbCl or CsCl. The effects of the presence of different cations on the rates of fumarate transport into *H. pylori* NCTC 11639 cells are shown in Table 3. The values measured for K^+ were lower than for the other four cations ($n = 3$).

The effects of pH on fumarate transport were examined by measuring rates at 20°C, 100-μM permeant, and different pH. Cells in PBS suspensions at pH 7 were centrifuged and the supernatants removed. The cells were resuspended in PBS solutions at the desired pH containing labeled fumarate, and the amount of the permeant transported was measured at a fixed timepoint of 30 sec. No significant changes in transport rates were observed for cell suspensions in PBS at pH between 5 and 9 ($n = 3$).

Inhibition of Fumarate Transport

The mechanisms of fumarate transport in *H. pylori* were characterized further by examining the effects on the

Table 2. Effect of amino acids on the rates of fumarate entry into strain NCTC 11639 *H. pylori* cells

Amino acid	Activity (% of control)
Alanine	75
Glycine	98
Isoleucine	77
Leucine	78
Valine	64
Cysteine	108
Methionine	111
Serine	106
Threonine	108
Proline	98
Phenylalanine	73
Tryptophan	68
Tyrosine	61
Arginine	93
Histidine	112
Lysine	103
Ornithine	104
Asparagine	104
Glutamine	93
Aspartic acid	85
Glutamic acid	84

Rates were measured in cells suspended in phosphate buffered saline, pH 7 and 1 mM amino acid, by the centrifugation through oil method at a fumarate concentration of 100 μM, 20°C and a timepoint of 30 sec. Values are quoted relative to the rates measured in solutions without any amino acids ($n = 3$). Activities are given as the median of the measurements expressed as percentage of the control. Errors were estimated at ±8%

Table 3. Effect of monovalent cations on the rates of fumarate transport into strain NCTC 11639 *H. pylori* cells

Cation	Rate (nmole/min/mg protein)
Li^+	58 ± 4
Na^+	48 ± 3
K^+	44 ± 3
Rb^+	72 ± 5
Cs^+	81 ± 6

Phosphate buffered saline (PBS) solutions were prepared with phosphate and chloride salts (150 mM) of different monovalent metals. Transport rates of cells suspended in these solutions were measured by the centrifugation through oil method at 20°C and a timepoint of 30 sec ($n = 4$).

transport of 100-μM fumarate at 20°C and a fixed timepoint of 30 sec, of several inhibitors, uncouplers and ionophores known to interfere with different transport processes in cells. The two protocols employed yielded similar results suggesting that in the time scale of the assay the effects were not reversible. The results are summarized in Table 4 ($n = 3$). Statistical analysis of the data served to ascertain which compounds had significant effects on fumarate transport. The inhibitor of electron transport in mitochondria, sodium cyanide, decreased fumarate transport. No inhibition was observed with the alkylating agent iodoacetamide and the nonspecific sulfhydryl *p*-chloromercuribenzoate.

Table 4. Effect of potential inhibitors on the rates of fumarate into strain NCTC 11639 *H. pylori* cells

Inhibitor	Activity (% of control)
Sodium fluoride (20 mM)	84
Sodium azide (10 mM)	72
Sodium cyanide (2 mM)	47
Iodoacetamide (2 mM)	105
p-Chloromercuribenzoate (2 mM)	111
Amiloride (10 μM)	99
Oubain (1 mM)	94
Furosemide	92
2,4-Dinitrophenol (2 mM)	52
Carbonyl cyanide *m*-chlorophenyl hydrazone (2 μM)	44
Oligomycin (2 μM)	80
Valinomycin (2 μM)	72
Monensin (2 μM)	49
Nigericin (6 μM)	48

Rates were measured in cells suspended in phosphate buffered saline, pH 7 by the centrifugation through oil method at a fumarate concentration of 100 μM, 20°C and a timepoint of 30 sec. Values are quoted relative to the rates measured in solutions without any inhibitors ($n = 3$). Activities are given as the median of the measurements expressed as percentage of the control. Errors were estimated at ±10%

The presence of the uncoupler DNP decreased the rates of fumarate transport; and the protonophore CCCP which allows protons to reach electrochemical equilibrium across the membrane [16], also inhibited fumarate transport (Table 4).

No changes in transport rates were observed in cells incubated with amiloride, furosemide or ouabain, inhibitors of sodium transport, the Na^+,K^+,$2Cl^-$ carrier, and the Na^+,K^+-ATPase, respectively (Table 4). Oligomycin is an inhibitor of H^+-ATP synthase and H^+ translocation in mitochondria and in some microorganisms such as *Rhodospirillum rubrum* and to a lesser extent *E. coli* [19]. Valinomycin is a ionophore that makes membranes permeable to cations, with preference for K^+ and is able to collapse the transmembrane potential without altering directly the pH gradient [15, 50]. The ionophores monensin and nigericin have high selectivity for Na^+ and K^+, respectively, promoting the exchange of H^+ for Na^+ (monensin) or H^+ for K^+ (nigericin), and causing the H^+ and cation gradients to equalize. These reagents are used to dissipate the pH gradient across the membrane, but maintaining the membrane potential [15, 23]. The transport of fumarate was reduced in the presence of monensin and nigericin, but oligomycin and valinomycin did not have significant effects (Table 4).

The effects of DNP, nigericin and valinomycin on

Table 5. Effects of inhibitors on the rates of fumarate transport into strain NCTC 11639 *H. pylori* cells at different pH

Inhibitor	Rate (nmole/min/mg protein)		
	pH 5.5	pH 7.0	pH 8.5
Control	43 ± 4	42 ± 4	42 ± 4
2,4-Dinitrophenol (2 mM)	29 ± 2	26 ± 2	22 ± 2
Nigericin (6 μM)	37 ± 3	31 ± 3	26 ± 3
Valinomycin (2 μM)	42 ± 3	34 ± 3	24 ± 3

Cells were incubated for 30 min with the inhibitors at concentrations showed in brackets in PBS solutions at pH 7.0. The cells were centrifuged and resuspended in PBS at pH 5.5, 7.0 or 8.5 containing 100 μM fumarate for 30 sec. Transport rates of cells suspended in these solutions were measured by the centrifugation through oil method at 20°C ($n = 2$).

fumarate transport in the presence of pH gradients were studied at pH 5.5, 7.0 and 8.5. The rates of transport at the different pH are given in Table 5. The rates of transport decreased linearly with pH, and the slopes of these lines were −2.3 ± 0.4, −3.7 ± 0.4, −6.0 ± 0.6 and 0 nmole/min/mg protein/pH unit, for the samples with DNP, nigericin, valinomycin and without inhibitors, respectively.

Incorporation of Fumarate Carbon Atoms to the Cellular Mass

Analyses of the incorporation of radioactive fumarate into the cellular mass showed that 53 ± 10% of the label incorporated went into lipids, 29 ± 10% into proteins, 10 ± 5% into DNA and 8 ± 4% into cell-wall debris. The ratio of absorbances at 260 and 280 nm of the extracted DNA samples was 1.75 ± 0.05, indicating pure samples [2].

Discussion

Fumarate Transport Systems

Bacteria are capable of regulating the influx of nutrients and ions across the membrane by specific carrier proteins [31]. Significant rates of fumarate transport were observed at micromolar permeant concentrations employing the centrifugation through oil method. The transport had saturable kinetics (Fig. 2), was reversible, depended on temperature (Fig. 3), and was affected by the presence of dicarboxylic acids (Table 1) and inhibitors (Table 3). Inhibition of fumarate reductase by oxantel [39] did not affect the rates of fumarate influx or efflux, indicating that the transport was not coupled to the last step of anaerobic respiration, and that the label was transported as fumarate. The difference between the kinetic parameters for the influx and efflux of fumarate suggested an asymmetry in the intake and release transport systems. These results together with the data from time courses (Fig. 1) provided evidence that influx and efflux of fumarate at low concentrations from *H. pylori* cells was a carrier-mediated secondary transport with the driving force supplied by the chemical gradient of the anion [28, 41].

The influx of fumarate at millimolar concentrations showed nonsaturable kinetics suggesting the presence of leakage, that is, pathways other than the transport system observed at micromolar permeant concentrations [46]. Fumarate leakage became significant at high extracellular permeant concentrations and depended on the age of the cells, indicating that it was associated with the general morphological changes in *H. pylori* cell wall that leads to the conversion of bacillary forms to coccoid forms [7, 45]. Thus the data suggested that fumarate leakage may arise from a permeabilization of the bacterial membrane; although it is possible also that a low-affinity, high-capacity transport system expressed late in the growth phase of the cells could cause or contribute to the leakage.

Substrate Specificity of the Transport of Fumarate

Competition studies of fumarate influx with carboxylic acid anions served to ascertain the specificity of the transport system (Table 1). Fumarate entry was inhibited by the presence of other dicarboxylates, and was either stimulated or not affected by mono- or tricarboxylates suggesting that it is carried into the cells by one or several dicarboxylic acid transporters. The strongest inhibition of transport was observed with maleate (''*cis*-fumarate'') indicating that the system lacked specificity for the absolute configuration of fumarate. In particular, succinate inhibited competitively the influx of fumarate with a K_i of 1.7 mM (Fig. 4). Broad specificity for dicarboxylates has been found in *A. vinelandii* [42], *B. japonicum* [24], *B. subtilis* [16], *E. coli* [27], *E. aerogenes* [24], *P. putida* [9], rhizobia [14, 32], and *S. typhimurium* [26] indicating that *H. pylori* shared in a property ubiquitous among bacteria.

Dicarboxylates inhibited transport by 25–38%, with the exception of α-ketoglutarate (Table 1). Similarly to the mitochondrial dicarboxylic anion transporter, α-ketoglutarate did not compete with fumarate for entry into *H. pylori* cells. This characteristic is also found in the *B. subtilis* [16], *E. coli* [27], and *R. leguminosarum* [14] carriers; whereas influx of succinate into *P. putida* [9] and *S. typhimurium* [26] is inhibited by the presence by α-ketoglutarate.

Fumarate/Succinate Antiport

Figure 5 shows that the rates of transport of fumarate increased with the amount of succinate loaded into the cells, indicating that the entry of fumarate is facilitated by the efflux of succinate and suggesting the presence of an antiport fumarate/succinate system. The anaerobic fumarate transport in *E. coli* is stimulated by preloading bacterial cells with dicarboxylates, and the proposed physiological role of this system is a fumarate/succinate antiport under conditions of fumarate respiration [13]. Anaerobic fumarate respiration is present in *H. pylori* and its inhibition leads to cell death [33, 39]. The fumarate/succinate antiport would maintain charge neutrality by exchanging dicarboxylic anions and at the same time export succinate, the principal product of fumarate respiration, thus facilitating this mode of respiration in *H. pylori.* This type of exchange process which does not consume energy for substrate/product translocation is important for fermentative bacteria which generate limited amounts of metabolic energy from their catabolism [41]. This exchange mechanism would be very appropriate for *H. pylori* in which several fermentative pathways have been identified [33, 36, 37].

Two types of dicarboxylic acid transport systems have been found in bacteria. High-affinity systems with K_M between 1 and 30 μM have been observed in *E. coli* grown aerobically [13], *P. putida* [9], and cells and bacteroids of *B. japonicum* and cowpea *Rhizobium* [32]. Low-affinity systems with K_M greater than 100 μM have been identified in *B. subtilis* [16], *E. coli* grown anaerobically [13], and *E. aerogenes* [24]. Biphasic kinetics for the uptake of succinate revealed the presence of high- and low-affinity carboxylate transport systems in *B. japonicum* [24], and *S. typhimurium* [26]. The mitochondrial dicarboxylate carrier is a low-affinity transporter with K_M of 200 μM for malate and 1.2 mM for succinate [29]. The low-affinity fumarate transport system of *H. pylori* may reflect the ability of the bacterium to obtain fumarate readily from amino acids [33, 34], and thus would not have an essential requirement for uptake of this anion. At the same time the dicarboxylate transporter could be employed for the efflux of succinate produced by anaerobic respiration in exchange for other extracellular substrates.

Effects of Amino Acids

The data shown in Table 2 indicated that with the exception of glycine, significant inhibition of fumarate transport rates occurred only in the presence of aliphatic and aromatic amino acids. At pH 7 these amino acids will be zwitterions with hydrophobic side chains which are neither charged nor form hydrogen bonds, suggesting that molecules with these characteristics are able to interact with the fumarate transporter or transporters and thus cause inhibition of dicarboxylate entry into cells. The lack of effects of monocarboxylates on fumarate transport rates presented in Table 3 can be interpreted similarly. At neutral pH these molecules are charged at the carboxylate end, and acetate, lactate and pyruvate also contain hydrophobic methyl moieties. The absence of inhibition observed in the presence of formate, acetate and glyoxylate could be attributed to their small size; and the hydrogen bond forming moieties of lactate and pyruvate may be responsible for lack of effect of these carboxylic acids on fumarate transport.

Effects of Monovalent Cations

There is a gradation of the basic physicochemical properties of the alkali cations from Li^+ to Cs^+. However, ionic fluxes of monovalent cations through ion-selective carriers or channels indicate that the rates of diffusion of these ions do not depend exclusively on their size [21], but on the size of the hydrated ion and the strength of the binding of water to the ion [47].

The rates of fumarate entry into *H. pylori* cells depended on the alkali cation in the buffer, with the lowest values measured in the presence of K^+ and Na^+ (Table 2). Since selection requires interaction, an explanation of these observations needs to consider cation-carrier interactions. The effects of aliphatic and aromatic amino acids and monocarboxylates on fumarate transport were interpreted in terms of interactions of charged and neutral moieties in those molecules with the carrier or carriers.

Eisenman [10] proposed that cation selectivity would arise whenever hydration energy and binding energy (to a colloid, channel, carrier, etc.) depended differently on the ionic radius of the alkali cations. Eleven sequences (''Eisenman sequences'') common in chemistry and biology were predicted based on a simple model with a selectivity maximum for one of the cations and a decrease in the free energy for the ions with larger or smaller size [11]. Another analysis carried out to interpret the interactions of hydrated alkali cations with sites yielded series of affinities (''polarizability sequences'') of the six cations ordered from the most to the least preferred ion, according to the type of interaction [5]. The rates of fumarate transport measured in the presence of each one the six alkali cations (Table 2) follow within experimental error the polarizability sequence $Cs^+ > Rb^+ > Li^+ > K^+ > Na^+$, which is the reverse of the Eisenman sequence IX ($Na^+ > K^+ > Li^+ > Rb^+ > Cs^+$). The results in Table 2 suggested that fumarate transport took place with at least one bound monovalent cation, and the observations could be explained by carrier-cation interactions together with the smaller energies required to strip the hydration shell of Rb^+ and Cs^+. This

interpretation does not exclude other possible effects such as changes in membrane potential and pH, although no significant changes in transport rates were observed between pH 5 and 9.

Effects of Potential Inhibitors

The dependence of fumarate influx rates on alkali metal ions (Table 3) suggested that the transport of the anion into *H. pylori* cells was linked to monovalent cations. The inhibition of fumarate influx by dissipation of the sodium transmembrane gradient with monensin (Table 4), supported this interpretation. Since fumarate entry was not inhibited by the sodium transport inhibitor amiloride (Table 4), the results would suggest that the system transported fumarate-alkali cation complexes. In this case to maintain an electroneutral exchange process in the fumarate/succinate antiport the efflux of succinate would have to take place with succinate-cation complexes. Absence of inhibitory effects by furosemide (Table 4) indicated that the fumarate carrier or carriers were different from the Na^+,K^+,$2Cl^-$ carrier.

Although no significant changes in fumarate transport rates in *H. pylori* were observed between pH 5 and 9, nor for gradients established using extracellular pH between 5.5 and 8.5 (Table 5), the inhibition of transport by carbonyl cyanide *m*-chlorophenyl hydrazone (Table 4), suggested that the transmembrane H^+ gradient may be a factor in the entry of fumarate into *H. pylori,* since the protonophore would induce proton electrochemical equilibrium across the membrane. Comparison of the lack of effect of valinomycin with the inhibition by nigericin (Table 4) led to the same conclusion, because both ionophores would dissipate the K^+ gradient, but nigericin would affect also the H^+ gradient. This interpretation was supported also by the inhibition of fumarate influx by cyanide and DNP (Table 4), and by the effects on fumarate entry of pH gradients established in the presence of inhibitors. These effects on transport rates can be described by the slopes of plots of rates vs pH (Table 5). In the presence of DNP, nigericin, and valinomycin the rates were affected least by pH changes in the presence of DNP, which dissipates the H^+ gradient, and affected most in the presence of valinomycin, which only dissipates the K^+ gradient; the effects of nigericin, which dissipates both gradients was in between those of the other two compounds. Uncouplers reduced rates of fumarate entry indicating that the transport mechanism depended on an energized membrane or on utilization of ATP. Since transport of fumarate was not affected by fluoride, oubain, or oligomycin inhibitors of phosphatase activities, Na^+,K^+-ATPase, and H^+-ATP synthase, respectively, it is unlikely (but not impossible) that ATP was directly utilized by the transport mechanism. Dicarboxylic anion transport driven by an energized membrane has been observed for rhizobia [14, 17, 32, 43], *B. japonicum,* and *E. aerogenes* [24].

The alkylating agent iodoacetamide and the nonspecific sulfhydryl reagent *p*-chloromercuribenzoate did not inhibit fumarate uptake by *H. pylori* (Table 4) suggesting that sulfhydryl groups do not have an influence on the functioning of the carrier or carriers. The *H. pylori* transporters appeared to be different from those in *B. subtilis* [16], *E. coli* [27], *P. putida* [9], rhizobia [17, 43], *S. typhimurium* [26] and the mitochondrial dicarboxylate carrier [3], which are inhibited by sulfhydryl reagents.

Incorporation of Radiolabel to the Cellular Mass

The incorporation of radioactive carbon atoms from fumarate into the biomass of the bacterium indicated that besides the utilization of this metabolite in fumarate respiration for bioenergetic purposes, the carbon skeleton of the dicarboxylate was employed also in biosynthesis. Most of the incorporated label went into lipid (53%) and protein synthesis (29%), suggesting a distribution of the fumarate carbon skeleton through intermediates of the Krebs' cycle.

Carboxylic Acid Transporters of *H. pylori*

Analyses of the complete *H. pylori* genome revealed the presence of four open reading frames coding for proteins with high sequence similarities to carboxylic acid transporters of other microorganisms [48]. The sequence HP0143 codes for a protein with 57.7% similarity to the α-ketoglutarate/malate translocator (*SODiT1*) of *Haemophilus influenza.* The sequences HP1091, HP0724 and HP0140 code for proteins with 69.7% similarity to the α-ketoglutarate permease (*kgtP*), 75.3% similarity to the anaerobic C_4-dicarboxylate transport protein (*dcuA*) and 78.2% similarity to the L-lactate permease (*lctP*) of *E. coli,* respectively [48].

The significant similarity between the *H. pylori* and the anaerobic *E. coli* C_4-dicarboxylate transporters agreed with the low affinity of both systems [13], although maximal transport rates in *H. pylori* were closer to those of the aerobic system of *E. coli* [27], and the mitochondrial transporter. The presence of specific lactate and α-ketoglutarate permeases to transport these metabolites into *H. pylori* cells correlated with the observed lack of competition for entry into the bacteria between fumarate and lactate or α-ketoglutarate. The data on substrate specificity and the effects of amino acids on fumarate entry provided some insights into the mechanisms of permeant selection in *H. pylori* transport.

This investigation demonstrated the presence of a fumarate transport system in *H. pylori* with some properties closer to the mitochondrial dicarboxylate carrier

and the anaerobic *E. coli* fumarate transporter than to any of the other bacterial systems that have been studied, with the possible exception of *B. subtilis.*

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