The Membrane Potential in a Cytochrome-deficient Species of *Bacteroides*: Its Magnitude and Mode of Generation

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(Received 2 June 1986; revised 7 July 1986)

The lipophilic cations butyltriphenylphosphonium (BTTP⁺), tetraphenylphosphonium (TPP⁺) and triphenylmethylphosphonium (TPMP⁺) were taken up into cells of *Bacteroides amylophilus* H18 under anaerobic conditions. Uptake was dependent on the presence of maltose together with both HCO₃⁻ and Na⁺; it was at a maximum at concentrations of ≥ 20 mM-HCO₃⁻ and ≥ 2 mM-Na⁺. The addition of 2-(n-heptyl)-hydroxyquinoline-*N*-oxide (HpHOQnO) or of an uncoupler of oxidative phosphorylation, or air, resulted in efflux of the lipophilic cations. From the binding behaviour and the physiological effects of the lipophilic cations, a membrane potential ($\Delta\psi$) of 140 mV was estimated. There was no detectable Δ pH at an external pH of 6.7. The cytoplasmic Na⁺ concentration was estimated to be 0.2 mM, indicating that *B. amylophilus* can maintain a Na⁺ concentration gradient equivalent to at least 150 mV. Variation in the external Na⁺ concentration (2 to 180 mM) had little influence on $\Delta\psi$. High external concentrations of the fermentation products acetate, formate and succinate had little effect on the growth rate and the $\Delta\psi$. The cytoplasmic ATP concentration decreased rapidly on addition of HpHOQnO oxide or of an uncoupler. The maximum internal ATP concentration was only maintained at an external concentration ≥ 2 mM-Na⁺. The NADH:fumarate reductase activity of vesicles of *B. amylophilus* was associated with alkalization of the suspension medium. The amount of H⁺ taken up was in excess of the expected amount of scalar H⁺ and was partially sensitive to uncoupler. It is concluded that the $\Delta\psi$ was generated via H⁺ translocation driven primarily by the cytochrome-deficient NADH:fumarate reductase system. The transmembrane Na⁺ gradient could be supported via the action of a Na⁺/2 H⁺ antiporter.

INTRODUCTION

Several forms of membrane-associated free energy conservation, coupled to ATP synthesis or transport processes, occur in facultatively and obligately anaerobic chemoheterotrophic bacteria. The primary process for generating Δp (Mitchell, 1966) may be electron transport to a number of alternative electron acceptors (Thauer *et al.*, 1977), ATP hydrolysis catalysed by a membrane-bound proton-translocating ATPase (Maloney, 1983; Harold & Heefner, 1981), or efflux of fermentation end-products (Ten Brink & Konings, 1982). One of these latter processes

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DTE, dithioerythritol; HpHOQnO, 2-(n-heptyl)-hydroxyquinoline-*N*-oxide; TTFB, 4,5,6,7-tetrachloro-2'-trifluoromethylbenzimidazole; SF 6847, 3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile; TCS, tetrachlorosalicylanilide; TPP⁺, tetraphenylphosphonium cation; BTTP⁺, butyltriphenylphosphonium cation; TPMP⁺, triphenylmethylphosphonium cation; Δp , protonmotive force (proton electrochemical gradient in mV); $\Delta\psi$, membrane potential; Δ pH, transmembrane pH gradient; PMSF, phenylmethylsulphonyl fluoride.

is catalysed by a fumarate reductase system, which typically comprises a dehydrogenase, menaquinone or desmethylmenaquinone, cytochrome(s) *b* and fumarate reductase (Stouthamer, 1980; Konings & Michels, 1980; Kröger, 1978). 'Fermentative' electron transport complexes of this type may resemble an early stage in the evolution of membrane-bound energy transduction systems (Wilson & Lin, 1980; Gest, 1980).

Bacteroides amylophilus is a strict anaerobe and is prominent in the rumen microflora (Hobson & Wallace, 1982). This bacterium possesses a simple carbohydrate catabolic pathway, with starch or maltose as sole substrate, through which glycolysis is coupled via a phosphoenolpyruvate carboxykinase catalysed step to the formation of fumarate, which is in turn reduced by the membrane-associated electron transport chain to yield succinate. Succinate, together with approximately equal amounts of acetate and formate, is released into the growth medium (Hobson & Summers, 1967). The electron transport chain of *B. amylophilus* includes an NADH dehydrogenase and fumarate reductase, but not detectable cytochromes, menaquinone or desmethylmenaquinone (Reddy & Bryant, 1977; Wetzstein & Gottschalk, 1985). Despite the absence of cytochromes the growth yields are amongst the highest recorded for bacteria growing fermentatively under anaerobic conditions (Hobson & Summers, 1967; Jenkinson & Woodbine, 1979), and there is a fumarate reductase system of relatively high activity (Wetzstein & Gottschalk, 1985). *B. amylophilus* is therefore a valuable subject for comparative study in relation to both the overall physiological role of the fumarate reductase systems and their mechanism of action.

We report here the first quantitative studies of the bioenergetics of a strict anaerobe with the special features of *B. amylophilus*. We have addressed ourselves primarily to the questions of what is the composition and magnitude of Δp in cells of *B. amylophilus*, how it is generated, and under what conditions it is stably maintained.

METHODS

Growth and preparation of cells. Cells of *B. amylophilus* H18 (ATCC 29744, DSM 1361) were grown anaerobically at 39 °C in a mineral medium and harvested as described by Wetzstein & Gottschalk (1985). For measurements of the uptake of lipophilic ions, cells were (unless otherwise stated) washed once in 20 mM-HEPES/NaOH (pH 6.7), 74 mM-NaHCO₃, 2 mM-MgCl₂, 1 mM-DTE, 0.17 mM-titanium(III)citrate (under 100% CO₂), resuspended in the same buffer plus 50 µg chloramphenicol ml⁻¹ and 0.1 mM-PMSF to a final (equivalent) OD₆₀₀ of 140 ± 20, and stored under 100% CO₂ in Hungate tubes on ice until use. Internal volume estimates for the cells were made as the sucrose-impermeable space [1.1 µl (mg dry wt)⁻¹] and the inulin-impermeable space [2.5 µl (mg dry wt)⁻¹], respectively, using a method similar to that described by Sorgato *et al.* (1978).

Preparation of membrane vesicles using a French press. Bacteria were harvested from 1 l of culture, resuspended in 12 ml of anaerobic buffer containing 20 mM-HEPES/KOH, pH 6.7, 10 mM-MgCl₂, 2.5 mM-DTE, 0.1 mM-PMSF and 0.33 mM-titanium(III)citrate under 100% N₂, and then passed through a French press at 69 MPa. The internal volume of the vesicles [1.6 µl (mg vesicle protein)⁻¹] was determined as the sucrose-impermeable space as described for whole cells. Protein was determined as described by Wetzstein & Gottschalk (1985).

Preparation of spheroplasts for lipophilic cation binding studies. Cells from 1 l of culture were harvested under anaerobic conditions as described above and resuspended in 18 ml 10 mM-Tris/HCl, pH 7.7, 0.5 M-sucrose, 0.1 mM-PMSF. A stock solution of EDTA-Tris, pH 8.0 was added to give a final concentration of 10 mM and after a 10 min incubation at 20 °C the suspension was centrifuged at 48000 g for 30 min. The pellet was resuspended in the original buffer, to which lysozyme was added (100 µg ml⁻¹). After incubation for 60 min at 30 °C the suspension was cooled on ice, diluted 1:1 with distilled water and carefully homogenized. Finally, the spheroplast suspension was centrifuged for 15 min at 48000 g and the pellet was resuspended in 20 mM-HEPES/NaOH, pH 6.7, 74 mM-NaHCO₃, 0.25 M-sucrose, and stored on ice. Spheroplast preparations were checked microscopically. NADH:fumarate reductase activity was negligible (Wetzstein & Gottschalk, 1985).

Estimation of the magnitudes of the components of Δp . Ion selective electrodes were constructed as described by Alefounder *et al.* (1981) and McCarthy & Ferguson (1983). The uptake of lipophilic ions was measured in water-jacketed glass vessels maintained anaerobically at 39 °C. Attempts to measure the uptake of SCN⁻ into membrane vesicles during NADH:fumarate reductase activity were made using either 3 ml of the reaction medium described by Wetzstein & Gottschalk (1985) or the same medium with NADH replaced by an NADH-regenerating system (cf. McCarthy & Ferguson, 1983). In further experiments ATP (2 mM) was added and NADH (or the NADH-regenerating system) was omitted. Vesicle protein was used in the range 2 to 10 mg per assay and the final concentration of SCN⁻ was 40 µM.

Measurements of the distribution of [7-¹⁴C]benzoate (cf., e.g. Ramos *et al.*, 1979) were made at 39 °C using a water-jacketed flow-dialysis cell constructed from glass (Ochs, Bovenden-Lengler, FRG). The upper and lower reaction chambers (0.9 cm in diameter with volumes of 1.5 and 0.15 ml, respectively) were separated by dialysis tubing. Anaerobic conditions were maintained in the reaction chamber and the buffer reservoir by continuous gassing with oxygen-free CO₂. Fresh growth medium lacking maltose was pumped through the lower chamber at 1 ml min⁻¹. Fractions (1 ml) were analysed with a Beckman LS 7500 scintillation counter. The upper chamber contained, in a total volume of 0.5 ml, fresh growth medium without maltose together with the equivalent of 8 mg dry wt cells, 0.2 mM-titanium(III)citrate and 1.4 µCi (51.8 kBq) [7-¹⁴C]benzoic acid (96 µM). Maltose (5 mM) was added at appropriate times. The pH in the upper chamber was monitored using a standard pH electrode (Metrohm, Herisau, FRG).

Determination of intracellular Na⁺. A growing culture (0.5 l) was supplemented at an OD₆₀₀ of 0.5 with 10 µCi (370 kBq) ²²NaCl, and with 5 µCi (185 kBq) [¹⁴C]sucrose (plus 1 mM cold sucrose to minimize binding of label) at an OD₆₀₀ of 1.0. Cells were subsequently harvested by centrifugation, resuspended in about 20 ml of fresh growth medium and disrupted by sonication. After addition of a few crystals each of DNAase, RNAase and lysozyme the extract was centrifuged at 165 000 *g* in a Sorvall OTD 65 centrifuge for 3 h. The supernatants obtained before and after cell breakage were analysed for radioactivity.

Measurement of pH shifts generated by membrane vesicles. Membrane vesicles were prepared by the French press procedure described above except that they were finally resuspended in the buffer used for pH shift measurements (see later text and legend to Fig. 4). pH shifts were measured using a Russell type 757 pH electrode. N₂ (100%) was pumped continuously over the reaction mixture which was maintained at 39 °C.

Determination of membrane-associated enzyme activities and of cytoplasmic ATP. NADH:fumarate reductase (EC 1.3.99.1) activity was determined as described by Wetzstein & Gottschalk (1985). The membrane-bound ATPase activity was assayed either as described by Vogel & Steinhart (1976) or by determination of the rate of P_i release. All measurements were made under 100% N₂ in Hungate tubes at 39 °C. In the second method, the assays contained, in a final volume of 625 µl, 83 mM-HEPES/KOH, pH 8.0, 1.6 mM-MgCl₂, 0.5–2.0 mg vesicle protein and 2 mM-ATP-KOH, pH 7.0. At appropriate times 100 µl samples were taken, mixed with 100 µl ice-cold 1 M-trichloroacetic acid and analysed for P_i (Taussky & Shorr, 1953). ATP was determined using luciferin/luciferase (Schienz *et al.*, 1981).

Radiochemicals. [7-¹⁴C]Benzoic acid [specific activity 29.4 mCi mmol⁻¹ (1.088 GBq mmol⁻¹)] was obtained from New England Nuclear, FRG. [¹⁴C]Sucrose [584 mCi mmol⁻¹ (21.61 GBq mmol⁻¹)], ²²NaCl [500 mCi (mg Na)⁻¹; 18.5 GBq (mg Na)⁻¹], (hydroxy[¹⁴C]methyl)inulin [13.8 mCi mmol⁻¹ (510.6 MBq mmol⁻¹)] and ³H₂O [25 µCi mmol⁻¹ (925 MBq mmol⁻¹)] were obtained from Amersham Buchler, FRG.

RESULTS

Uptake of lipophilic cations (BTTPP⁺, TPMP⁺ or TPP⁺) into whole cells of B. amylophilus

This was measured using the constructed ion selective electrodes. After calibration of the electrode, cells were added to the reaction medium (Fig. 1). This resulted in an uptake of TPP⁺ consistent with the generation of a Δψ (cell interior negative). The characteristics of uptake of the lipophilic cations were similar to those observed when the cells were suspended in growth medium. HCO₃⁻ was essential for the uptake which was maximal at external concentrations of HCO₃⁻ ≥ 20 mM (not shown).

HpHOQnO, at the minimum concentration required to give substantial inhibition of the NADH:fumarate reductase, induced nearly complete release of the accumulated TPP⁺ (Fig. 1). Further release was elicited by the uncoupler TTFB (Fig. 1). The final displacement of the electrode reading from its initial value is attributed to the dilution of the cation caused by the addition of cells and the non-specific binding of TPP⁺. Complete dissipation of Δψ was also induced by low concentrations (≤ 5 µM) of any of the uncouplers TTFB, FCCP, TCS or SF 6847 alone (not shown).

None of the following ionophores were found to have any effect upon the uptake of lipophilic cations by *B. amylophilus*, possibly because they cannot permeate the outer membrane/cell wall of this species: monensin, valinomycin or valinomycin plus nigericin [each at 1 µg ionophore (mg cell protein⁻¹)].

The kinetics and final extents of uptake were similar for all three cations studied. As in *Escherichia coli* (cf. Muratsugu *et al.*, 1979), the permeability of the cytoplasmic membrane (and/or cell wall) to the lipophilic cations might be a limiting factor in the uptake process, since a preincubation of the concentrated cell suspension with 5 mM-EDTA-Tris, pH 7.0, for 5 min at

Fig. 1

Fig. 2

Fig. 1. Uptake of TPP^+ by *B. amylophilus* under anaerobic conditions. The reaction vessel contained a suspension mixture (10 ml) that contained 20 mM-HEPES/NaOH, pH 6.7, 70 mM- NaHCO_3 , 6 mM-maltose and $34 \mu\text{M}$ - $\text{Na}_2\text{S}_2\text{O}_4$ under a flow of 100% CO_2 that had been passed through a heated copper column and then through water before being fed through the reaction chamber. TPP^+ was added in increments of $2 \mu\text{M}$ for electrode calibration. Cells (equivalent to 19 mg dry wt) were pretreated with 5 mM-EDTA-Tris, pH 7.0, at room temperature for 5 min and then added as indicated, followed by HpHOQnO and TTFB to give final concentrations of 5.7 and $1.1 \mu\text{M}$, respectively. The measurements were made at 39°C .

Fig. 2. Stimulation of BTPP^+ uptake by Na^+ . The assay conditions were as specified in the legend to Fig. 1, except that the corresponding K^+ salts were used instead of Na^+ salts during washing and resuspension of the cells as well as in the reaction medium, and the distribution of BTPP^+ rather than TPP^+ was determined. Cells (equivalent to $1.2 \text{ mg dry wt ml}^{-1}$) were added as indicated, followed by NaCl and finally by HpHOQnO to give concentrations of 20 mM and $5.1 \mu\text{M}$, respectively.

room temperature gave an accelerated uptake. Alternatively, a process of metabolic activation may have been at least partially responsible for the kinetics of uptake.

The reproducible generation of a stable $\Delta\psi$ was observed only when strictly anaerobic conditions were maintained during the harvesting, resuspension and storage of the cells. If a small quantity of air (up to 20 ml) was blown through the reaction chamber (at 10 ml min^{-1}) there was a rapid release of the accumulated cation equivalent to a reduction in $\Delta\psi$ of up to 80 mV. The stability of the cells during storage was also found to be much increased by PMSF, an effective inhibitor of the extracellular protease of *B. amylophilus* (Lesk & Blackburn, 1971).

One possible mechanism of generation of $\Delta\psi$ in *B. amylophilus* considered was the coupling of ion transport to ATP hydrolysis catalysed by a membrane bound ATPase. *N,N'*-Dicyclohexylcarbodiimide ($20 \mu\text{M}$), venturicidin [$1 \mu\text{g (mg cell protein)}^{-1}$] or sodium azide ($100 \mu\text{M}$), inhibitors which interfere with the activity of such ATPases, had no effect on the generation of $\Delta\psi$ by *B. amylophilus*, even when preincubated with cells for up to 2 h before the measurement of lipophilic cation uptake. However, when arsenate, which in *E. coli* reduces the intracellular concentration of ATP and thus inhibits ATP-dependent transport activities (Klein & Boyer, 1972), was added during a lipophilic cation uptake experiment there was an initial, rapid drop in $\Delta\psi$ of approximately 25 mV, followed by a very slow decay. Alternatively, after a

Table 1. Correction of the estimates of $\Delta\psi$ for binding of the lipophilic cations to cellular constituents

Cells of *B. amylophilus* from various batches were used for measurements at 39 °C with TPMP⁺, BTPP⁺ and TPP⁺ respectively, within 5 h of harvesting. The uncorrected estimates were derived by application of the Nernst equation assuming that there was no binding and that the lipophilic cations behave 'ideally'. For calculation of the corrected values the modified Nernst expression (Lolkema *et al.*, 1982) was used:

$$\Delta\psi = \frac{2.3 RT}{F} \log \left\{ \frac{(C_o/C_e + x - 1)}{[x(1 + K_i)]} \right\}$$

where x = fractional internal volume of cells; C_e = external concentration of cation after addition of cells; C_o = external cation concentration before addition of cells but corrected for dilution; and K_i = ratio of bound to free cation inside the cells. When $\Delta\psi = 0$, as in the binding experiments with heat-treated cells, this equation can be rearranged to give: $C_o/C_e = 1 + xK_i$. The slopes of the plots of $(C_o/C_e - 1)$ vs x are therefore equivalent to K_i . The value of x for a given amount of spheroplasts was assumed to be the same as that of the equivalent amount of cells used in the preparation of these spheroplasts. n , No. of determinations.

Lipophilic cation	Whole cells					Spheroplasts		
	Uncorrected $\Delta\psi$ (mV)	n	Corrected $\Delta\psi$ (mV)	n	K_i	Corrected $\Delta\psi$ (mV)	n	K_i
TPMP ⁺	179	17	103	7	16	140	5	5.3
BTPP ⁺	175	15	68	10	49	—	—	—
TPP ⁺	173	3	67	7	53	112	2	9

preincubation of 30 min, there was an initial uptake equivalent to a $\Delta\psi$ of 123 mV (calculated without correction for binding) which was then followed by a slow release of the cation. The time course and extent of this effect of arsenate did not, however, correlate well with the results of parallel measurements of the intracellular concentration of ATP (see below).

Estimation of the magnitude of $\Delta\psi$

If it is assumed that BTPP⁺, TPMP⁺ and TPP⁺ behave ideally and become distributed between the interior of the cells and the external aqueous phase according to the Nernst equation, values in the range 170–180 mV for $\Delta\psi$ can be calculated (Table 1) using an estimated internal volume of the cells of $1.1 \mu\text{l (mg dry wt)}^{-1}$ (sucrose-impermeable space).

Corrections for binding of the lipophilic cations to constituents of *B. amylophilus* cells were made using the analysis of Lolkema *et al.* (1982). The extent of binding of each of the lipophilic cations to whole cells was assessed using heat-treated cells and a wide range of cation concentrations. All plots of cation bound vs the free cation concentration were linear in the range $8 \mu\text{M}$ to 3 mM and had slopes of 27, 42 and $61 \text{ pmol (mg dry wt cells)}^{-1} \mu\text{M}^{-1}$ for TPMP⁺, BTPP⁺ and TPP⁺ respectively, as determined by linear regression analysis. The extent of binding at a given cation concentration was linearly dependent on the cell concentration in the range 0.8 to 4.2 mg ml^{-1} . The chosen range of cation concentrations used in the binding experiments included the highest concentrations which could be expected to be reached in the cytoplasm of *B. amylophilus*.

If all of the binding is assumed to occur within the cytoplasm (Table 1), very large corrections of the calculated $\Delta\psi$ become necessary. The binding data for each of the three cations were used to construct plots of $(C_o/C_e - 1)$ vs x (see legend to Table 1 and Lolkema *et al.*, 1982), the slopes of which are equivalent to the corresponding values of K_i defined in Table 1. Calculating $\Delta\psi$ on this basis yields drastically reduced values, particularly with BTPP⁺ and TPP⁺, for which binding is more extreme (Table 1).

Spheroplasts were found to be incapable of supporting an uncoupler-sensitive uptake of lipophilic cations. Light microscopy revealed the spheroplasts to be intact spherical bodies lacking any discernible outer membrane and cell wall components. Their inability to take up

lipophilic cations could be attributable to the loss of a maltose binding protein required for transport and the consequential lack of substrate for glycolysis. The extent of binding at given concentrations of both TPMP⁺ and TPP⁺ to spheroplasts was approximately 20% of that observed with an equivalent amount of heat-treated whole cells (Table 1). Thus a large fraction of the lipophilic cation bound to heat-treated whole cells was bound to the outer membrane, cell wall and components of the periplasmic space, and the extent of this binding was responsive to the external concentrations of the respective cations. This extra binding would therefore have led to an overestimation of the applicable K_i value since these external sites would not be exposed to the higher cation concentrations within the cytoplasm after uptake. The values of K_i determined with spheroplasts should yield closer approximations to the true $\Delta\psi$ values (Table 1). This approach is justified on the basis that the amounts of lipophilic cation bound to the sites removed by spheroplast preparation are small relative to the internal binding when low external concentrations of cation are used.

The growth of *B. amylophilus* was inhibited to varying degrees by all three cations. TPMP⁺ (10 μ M) increased the doubling time from approximately 50 min to 84 min. BTPP⁺ and TPP⁺ had much more drastic effects: above 15 μ M they not only inhibited growth but also caused swelling and finally lysis. The cations also inhibited the NADH:fumarate reductase activity of membrane vesicles; the inhibitory effect of TPMP⁺ was again considerably smaller than that of BTPP⁺ or TPP⁺. In the concentration range up to 1 mM, a (free) concentration which is unlikely to be exceeded within the cells under the described experimental conditions, TPMP⁺ inhibited NADH:fumarate reductase activity by less than 10%, whereas TPP⁺ inhibited by up to 40%. Finally, the estimated $\Delta\psi$ was diminished by less than 5 mV when the initial external concentration of TPMP⁺ was raised from 8 μ M to 40 μ M, whereas the $\Delta\psi$ estimated from BTPP⁺ uptake decreased by up to 36 mV.

Estimation of Δ pH

Stable uptake of [¹⁴C]benzoic acid was not observed with cells in growth medium. A transient apparent uptake, equivalent to the development of a Δ pH of maximum magnitude 0.7, occurred when maltose was added to cells in growth medium (minus substrate) at pH 6.4. [¹⁴C]Benzoic acid can therefore permeate the cells, and the data indicate that *B. amylophilus* does not support a stable Δ pH of readily measurable (i.e. ≥ 0.5) magnitude under normal growth conditions.

Influence of the ionic environment on the generation of $\Delta\psi$

Freshly harvested cells, washed and resuspended in a buffer in which all Na⁺ salts had been replaced by K⁺ salts, supported the generation of an apparent $\Delta\psi$ of approximately 100 mV (Fig. 2). NaCl, which is essential for the growth of *B. amylophilus*, added at 20 mM, stimulated a further uptake of cation equivalent to a $\Delta\psi$ of approximately 160 mV. A similar effect was induced by Na₂SO₄ but not by KCl. The addition of HpHOQnO induced almost complete release of the cation (cf. Fig. 1).

The internal concentration of Na⁺ in *B. amylophilus* in growth medium was 0.2 mM, which indicates that the cells are relatively impermeable to Na⁺ and can maintain a transmembrane Na⁺ concentration ratio of 1:450.

The effect of variations in the external Na⁺ concentration on $\Delta\psi$ was investigated. Cells in a low Na⁺ medium were added to two reaction media (plus the indicated extra additions) for the measurement of TPMP⁺ uptake (Table 2). $\Delta\psi$ was estimated for cells in a low Na⁺ medium containing various amounts of Na₂SO₄ in the presence of xylose to compensate for changes in osmotic strength. The increase from 2 to 100 mM-Na⁺ would be equivalent to an overall increase of 100 mV in the chemical potential component of the transmembrane Na⁺ electrochemical potential (given an unaltered internal Na⁺ concentration). The estimated $\Delta\psi$, however, was only reduced by a maximum of 37 mV. Not all of this reduction was attributable to the effects of the Na⁺ ions alone, but rather also to the effects of the SO₄²⁻ ions (compare the effects of NaCl, Na₂SO₄ and NaHCO₃ in Table 2). In conclusion, *B. amylophilus* can maintain a relatively unperturbed $\Delta\psi$ in the presence of an external Na⁺ concentration throughout the range 2 to 180 mM.

Table 2. *Effects of variations in the concentrations of Na⁺ and of fermentation products on $\Delta\psi$*

Experiments were done essentially as described in the legend to Fig. 1 using either a K⁺ (low Na⁺) buffer or a high Na⁺ buffer together with 1.2 and 1.8 mg dry wt cells and the indicated additions. Calculations of $\Delta\psi$ (cf. legend to Table 1) were made without corrections for binding. All measurements were made with cells which had been shown capable of maintaining the maximum $\Delta\psi$ under the conditions described in the legend to Fig. 1. Each experiment was done at least twice.

	Additions	Concentrations of additions (mM)	$\Delta\psi$ (mV)
K ⁺ buffer*	Na ₂ SO ₄ + xylose	1; 150	175
	Na ₂ SO ₄ + xylose	5; 150	176
	Na ₂ SO ₄ + xylose	20; 90	156
	Na ₂ SO ₄	50	138
	NaCl	75	135
Na ⁺ buffer†	—	—	174
	Xylose	150	172
	NaCl	75	149
	NaHCO ₃	75	167
	Sodium acetate	50	163
	Sodium formate	50	153
	Sodium succinate	50	142

* K⁺ buffer contained: 20 mM-HEPES/KOH, pH 6.8, 18.4 mM-KHCO₃, 6.4 mM-maltose, 0.15 mM-titanium(III)citrate.

† Na⁺ buffer contained: 20 mM-HEPES/NaOH, pH 6.8, 74 mM-NaHCO₃, 6.4 mM-maltose, 0.15 mM-titanium(III)citrate.

Effects of high external concentrations of fermentation products on $\Delta\psi$ and the growth rate

We investigated the effects of raised external concentrations of acetate, formate and succinate on both $\Delta\psi$ (Table 2) and the growth rate. Control experiments were done with NaHCO₃ and xylose, respectively. The fermentation products induced only small reductions in the estimated magnitude of $\Delta\psi$. Acetate, like NaHCO₃, had no effect on the specific growth rate up to a concentration of 100 mM, whereas succinate and formate gave decreases of 20% or 30%, respectively, in the specific growth rate at this concentration. However, even xylose (100 mM) resulted in a reduction of 37%, and thus the small effects of succinate and formate actually measured are not specific to these compounds. In conclusion, the minimal effects on $\Delta\psi$ and growth rate observed indicate that fermentation product efflux does not play a major role in $\Delta\psi$ generation.

Determination of the intracellular ATP concentration

During a typical set of experiments (Fig. 3) with a single preparation of cells the intracellular ATP concentration reached a maximum of approximately 7.5 mM and declined slowly with time (and thus with each successive experiment; see, e.g., the arsenate experiment). This does not, however, significantly affect the qualitative interpretation of the data. Inhibition of the NADH:fumarate reductase system with HpHOQnO induced a rapid drop in the intracellular concentration of ATP (and in the $\Delta\psi$, see Fig. 1), because this inhibition blocks the turnover of NADH and thus brings the central metabolic pathway of *B. amylophilus* to a halt (Wetzstein & Gottschalk, 1985). The addition of an uncoupler (TTFB) at first also resulted in a decline in the intracellular concentration of ATP, but the ATP concentration slowly recovered, perhaps as the result of an increased rate of substrate-level phosphorylation. Maintenance of the normal intracellular concentration of ATP, like the generation of $\Delta\psi$ (cf. Fig. 2), was dependent on the presence of Na⁺ (Fig. 3). Finally, arsenate caused a transient reduction in the intracellular concentration of ATP, in contrast to its effect on $\Delta\psi$.

Fig. 3

Fig. 3. Intracellular ATP concentration of *B. amylophilus* as estimated by the luciferin/luciferase assay. Cells ($3.2 \text{ mg dry wt ml}^{-1}$) were suspended in 2.7 ml fresh growth medium (lacking maltose) in an anaerobic reaction vessel, except in the experiment demonstrating the effect of NaCl (○) in which case the cells were washed and resuspended in the low Na^+ buffer described in the legend to Fig. 2. At zero time maltose was added (4.5 mM). Samples (0.3 ml) were removed at the times indicated, quenched with ice-cold perchloric acid and analysed for ATP. Further additions (arrowed) were made as follows with the final concentrations given in parentheses: HpHOQnO ($11 \mu\text{M}$; ▲); TTFB ($12.8 \mu\text{M}$; △); sodium arsenate (10 mM ; ■); NaCl (20 mM ; ○). The results of a control experiment to which no further additions were made are also shown (●). The temperature was 39°C .

Fig. 4

Fig. 4. Apparent H^+ -translocation associated with the NADH:fumarate reductase activity of membrane vesicles derived from *B. amylophilus*. French press membrane vesicles were added to give a protein concentration of 2.5 mg ml^{-1} to a reaction vessel containing 2.5 ml 50 mM-KSCN , 100 mM-KCl , $1.5 \text{ mM-glycylglycine}$, $4 \text{ mM-sodium fumarate}$ and $90 \mu\text{M-titanium(III)citrate}$ at $\text{pH } 6.5$. At the points indicated 58 nmol NADH was injected into the reaction medium inducing alkalinization (indicated by an upwards deflection of the pH electrode trace). For trace (b) TTFB was added ($20 \mu\text{M}$) before the NADH pulse. For trace (c) Triton X-100 was added (0.08%) before the NADH pulse. The temperature was 39°C .

Investigations of possible ion transport reactions of membrane vesicles derived from B. amylophilus

The preparation of inside-out vesicles from *B. amylophilus* exposes the NADH:fumarate reductase and a membrane-associated ATPase [with activities of 150 and $<15 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$, respectively] to the reaction medium (Wetzstein & Gottschalk, 1985).

Unsuccessful attempts were made to detect, using an ion selective electrode, SCN^- uptake into vesicles in response to $\Delta\psi$ generation during the hydrolysis of ATP or the activity of the NADH:fumarate reductase (cf., e.g., Sorgato *et al.*, 1978).

Measurements of pH changes associated with the NADH:fumarate reductase activity of vesicle preparations were made with a pH electrode (Fig. 4). The vesicles were suspended in a medium containing high concentrations of Cl^- and SCN^- , which are commonly used in such experiments to prevent the development of an electrical 'back-pressure' on H^+ translocation (see, e.g., Scholes & Mitchell, 1970). The oxidation of NADH by fumarate catalysed by the vesicles was itself expected to induce an alkalinization of the medium equivalent to 1 (scalar) H^+ per NADH. However, the alkalinization observed was equivalent to almost 2 H^+ (1.8 H^+ , mean of ten experiments) per NADH (Fig. 4, trace a). In the presence of an uncoupler (TTFB) (Fig. 4, trace b) the alkalinization was reduced to an amount equivalent to approximately 1.2 H^+ (mean of nine experiments). Finally, in the presence of 0.08% Triton X-100 (Fig. 4, trace c), which

inhibits the NADH:fumarate reductase by more than 90%, NADH induced the very slow development of a much smaller alkalization presumably attributable to the residual NADH:fumarate reductase activity. A trace very similar to trace (c) of Fig. 4 was also obtained when HpHOQnO [$0.1 \text{ nmol (mg vesicle protein)}^{-1}$] was added instead of Triton X-100. No pH shift was observed when NADH was added to vesicles in a medium lacking fumarate.

The mean uncoupler-sensitive alkalization of the medium measured in these experiments was equivalent to approximately 0.5 H^+ per NADH. If this extra uncoupler-sensitive alkalization was attributable to the inward translocation of protons, these data would indicate that up to 1 H^+ is translocated into the vesicles for every NADH molecule oxidized by the NADH:fumarate reductase.

When ATP (2 mM) was added to the vesicles (suspended in a medium identical to that defined in the legend to Fig. 4 except that fumarate was omitted) only the onset of a slow acidification which would be the expected result of the (scalar) ATP hydrolysis reaction was observed.

DISCUSSION

TPMP⁺ was found to be the lipophilic cation of choice for the estimation of $\Delta\psi$ in *B. amylophilus* because both the extent of binding of this ion and its inhibitory action were considerably less marked than those of BTPP⁺ and TPP⁺, respectively. Thus of the estimates of the magnitude of $\Delta\psi$ summarized in Table 1 we consider 140 mV to be the most reliable and likely to be the nearest to the true value. For comparison, measurements of the magnitude of $\Delta\psi$ supported by the fumarate reductase system of *E. coli* yielded rather lower values of 100 to 110 mV (Hellingwerf *et al.*, 1981). The exact magnitude of the ΔpH component in *E. coli* would, however, have been extremely difficult to measure since this was relatively small (Hellingwerf *et al.*, 1981).

The generation of a $\Delta\psi$ by *B. amylophilus* was dependent on maltose together with both HCO_3^- and Na^+ . The requirement for Na^+ is particularly remarkable and identifies *B. amylophilus* as a member of the hitherto rather small group of non-halophilic bacteria which require Na^+ for generation of $\Delta\psi$ (Hilpert *et al.*, 1984). However, the following evidence indicates that Na^+ is not directly involved in $\Delta\psi$ generation in *B. amylophilus*. First, the magnitude of $\Delta\psi$ was almost invariable over a range of external Na^+ concentrations of 2 to 180 mM. Second, the $\Delta\psi$ was rapidly and completely collapsed on addition of a classical uncoupler (cf. Tokuda & Unemoto, 1982). Third, membrane vesicles supported uncoupler-sensitive pH shifts which were Na^+ -independent and required NADH and fumarate. The relaxation rates of the observed pH shifts were unusually low. This may, however, be attributable to a very low H^+ permeability of the *B. amylophilus* cytoplasmic membrane. Why then are Na^+ and HCO_3^- required by *B. amylophilus* for the generation of $\Delta\psi$? The requirement for HCO_3^- is immediately explicable since fumarate is formed from phosphoenolpyruvate and HCO_3^- via oxaloacetate and malate. The most probable function of Na^+ is that it is involved in maltose uptake. The Na^+ dependence (Perski *et al.*, 1981) of methanogenic bacteria is apparently also not attributable to a direct involvement of this ion in $\Delta\psi$ generation (Blaut *et al.*, 1985).

Our data indicate that the primary mode of generation of $\Delta\psi$ in *B. amylophilus* is via H^+ translocation by the NADH:fumarate reductase system. There is no indication that an ion translocation mechanism coupled to the efflux of fermentation products contributes significantly to the generation of $\Delta\psi$. The effects of arsenate are complex, but it is evident that the reduction in $\Delta\psi$ induced by arsenate was not simply achieved via its effect on the intracellular ATP concentration, but rather via some other (unknown) mechanism. This is consistent with, but does not prove, the contention that ATPase-linked ion translocation does not play a primary role in generating $\Delta\psi$ in *B. amylophilus*.

In the fumarate reductase systems of other bacteria both a *b*-type cytochrome and menaquinone (or desmethylmenaquinone) are generally required for proper function (Macy *et al.*, 1975; Thauer *et al.*, 1977). In the only previous study of a fumarate reductase system in a bacterium which is naturally devoid of cytochromes (Faust & Vandemark, 1970), it was suggested that fumarate reduction was coupled to ATP synthesis in a cytochrome-deficient

strain of *Streptococcus faecalis* (but see Kröger, 1978). There is no evidence that the highly active cytochrome-deficient NADH:fumarate reductase of *B. amylophilus* drives ATP synthesis in whole cells. It might possibly function only to generate a $\Delta\psi$ and to maintain redox balance. If, as suggested by the pH shift data, the NADH:fumarate reductase of *B. amylophilus* translocated 1 H^+ per NADH oxidized, 4 H^+ would be translocated per maltose catabolized. The maintenance of the large Na^+ gradient could then be explained on the basis of an antiporter-catalysed exchange of 2 H^+ per Na^+ (cf. Padan *et al.*, 1981); the net single charge moved inwards in this way would be consistent with the observation that the Na^+ concentration gradient is approximately equivalent in free energy to the measured $\Delta\psi$.

How might the NADH:fumarate reductase of *B. amylophilus* fulfil the function of a generator of $\Delta\psi$ in the absence of cytochromes? Perhaps this apparently primitive system is closely related to the earliest evolved ion-translocating electron transport chains (Wilson & Lin, 1980; Gest, 1980). The later evolution of cytochromes may have allowed increased flexibility in the mechanism and stoichiometry of ion translocation.

We are grateful to Professor Dr H. U. Schairer for support and encouragement during a period of experimental work in the Cytogenetics Department of the GBF, and to Dr S. J. Ferguson for discussion. The experimental work in the Institute of Microbiology, the University of Göttingen, was supported by the 'Stiftung Volkswagenwerk'. J. E. G. M. was the holder of a European Molecular Biology Organisation Long Term Fellowship during the period 1982–1984.

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