A novel type of energetics in a marine alkali-tolerant bacterium

$\Delta\bar{\mu}_{Na}$-driven motility and sodium cycle

B.V. Chernyak, P.A. Dibrov, A.N. Glagolev, M.Yu. Sherman and V.P. Skulachev

Department of Bioenergetics, Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117234, USSR

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Motility of a marine alkali-tolerant bacterium, *Vibrio alginolyticus*, can be observed in the presence of high concentrations of a protonophorous uncoupler, CCCP. Motility in the CCCP-containing media is completely inhibited by decrease in extracellular [Na^+] or by monensin-induced increase in intracellular [Na^+]. A mutant has been selected that grows only in media supplemented with a substrate such as acetate requiring no $\Delta\bar{\mu}_{Na}$ to be transported into the cell. Motility of the mutant was found to be completely inhibited by CCCP. Cyanide, CCCP and vanadate added separately or in twos inhibit motility only partially. The three poisons added together completely paralyse the cells. In this inhibitor cocktail, arsenate can substitute for CCCP + vanadate; cyanide can be replaced by anaerobiosis. It is concluded that (i) $\Delta\bar{\mu}_{Na}$ rather than $\Delta\bar{\mu}_{H}$ powers the flagellar motor of *V. alginolyticus* in the presence of CCCP, and (ii) in addition to the Na^+-motive respiratory chain [Tokuda, H. and Unemoto, T. (1982) J. Biol. Chem. 257, 10007–10014] there is a vanadate and arsenate-sensitive oxygen-independent mechanism of $\Delta\bar{\mu}_{Na}$ generation, presumably an ion-motive ATPase. A suggestion is put forward that circulation of Na^+ can replace that of H^+ in *V. alginolyticus*, $\Delta\bar{\mu}_{Na}$ being formed by the Na^+-motive respiratory chain and utilized by Na^+-solute symporters, the Na^+-driven flagellar motor and maybe by a reverse ion-motive ATPase.

1. INTRODUCTION

It is shown that in bacteria living under neutral or acidic conditions as well as in mitochondria, chloroplasts and chromatophores, $\Delta\Psi$ and ΔpH components of $\Delta\bar{\mu}_{H}$ proved to be unidirectional, i.e. the pH in the compartment which charges positively is lower than that in the opposite one. $\Delta\bar{\mu}_{H}$ generated by proton pumps can be utilized to perform different types of chemical, osmotic or mechanical work [1,2]. Among them, extrusion of Na^+ from the bacterial cells, carried out by an Na^+/H^+-antiporter, looks like a particular case. $\Delta\bar{\mu}$ pNa formed was found to support accumulation of certain solutes and to buffer ΔpH across the cytoplasmic membrane (reviews [3,4]).

Such a concept meets with difficulties when we deal with alkalophilic or alkali-tolerant bacteria. Here $\Delta\Psi$ and ΔpH are oppositely directed when proton pumps transport H^+ from neutral cytoplasm to alkaline outer medium. Recently Tokuda and Unemoto [5] reported that a marine bacterium, *Vibrio alginolyticus*, extrudes Na^+ from the cell in a respiratory chain-dependent electrogenic fashion, the process being resistant to protonophorous uncouplers. In the same group, it was shown that $\Delta\bar{\mu}_{Na}$ is the driving force for uptake of α-aminoisobutyrate by these bacteria [6]. An indication was also obtained that transport of 19 amino acids and sucrose into *V. alginolyticus* cells is Na^+-dependent [5].

Abbreviations: $\Delta\bar{\mu}_{H}$ and $\Delta\bar{\mu}_{Na}$, electrochemical potential differences of H^+ and Na^+, respectively; $\Delta\Psi$, electric potential difference; ΔpH and ΔpNa, concentration differences of H^+ and Na^+, respectively; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone

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In this work, we studied energetis of *V. alginolyticus* cells using their motility as an intrinsic non-invasive probe for membrane energization. It has been revealed that there is a Na^+-dependent mechanism of motility which is sensitive to CCCP + monensin. CCCP and monensin added separately failed to paralyse bacteria. Inhibitor analysis showed that Na^+-dependent motility can be supported by at least two energy sources, one arrested by cyanide or anaerobiosis (Na^+-motive respiration) and another arrested by arsenate or vanadate (presumably, an ion-motive ATPase). A tentative scheme is postulated suggesting that *V. alginolyticus* possesses a 'sodium cycle', i.e. an energy coupling system that uses $\Delta\bar{\mu}_{Na}$, instead of (or in addition to) $\Delta\bar{\mu}_H$, as a convertible membrane-linked energy currency.

2. MATERIALS AND METHODS

In most experiments, we used the marine bacterium *V. alginolyticus* 138-2 which was the generous gift of Professor Hajime Tokuda. The CCCP-sensitive mutant was selected from *V. alginolyticus* wild strain isolated by one of us (A.N.G.). Bacteria were grown aerobically at 37°C on the medium used in [5] with 3% NaCl with or without 0.5% peptone and 0.5% yeast hydrolysate (media 1 and 2, respectively). At the late exponential phase of growth, the cells were sedimented by centrifugation at 20°C. The sediment was suspended in medium 1 or 2. To measure motility rate, we estimated the time required to a bacterium to swim the 25 μm distance without changing direction of movement. Measurements were carried out at room temperature using a phase contrast microscope. To calculate the motility rate, the average data on 10 cells were used.

3. RESULTS

In fig.1 data of a typical experiment on motility of *V. alginolyticus* are shown. Measurement of the rate of motility was begun at pH 7.5. Under these conditions, CCCP causes a 4-fold decrease in the motility rate. Alkalinization up to pH 9.0 reverses this effect. In the presence of monensin, CCCP induces complete inhibition of motility at any of two studied pH values.

In fig.2 motility rate is plotted *vs* CCCP concentration. One can see that the CCCP-resistant portion of the motility rate at pH 7.5 without monensin cannot be diminished by addition of excess CCCP (1×10^{-4} M) whereas in samples with monensin 1×10^{-6} M CCCP completely paralyses the cells. At pH 9.0, the latter concentration of CCCP increases up to 2×10^{-5} M apparently due to lowering of the level of protonated form of the uncoupler. It is remarkable that without monensin, 1×10^{-6} M CCCP (pH 7.5) and 2×10^{-5} M CCCP (pH 9.0) fail to stop motility. Under these conditions, the effect of higher CCCP concentrations may be explained, at least partially, by inhibition of respiration, which was revealed in polarographic measurement of oxygen consumption by the bacteria (not shown).

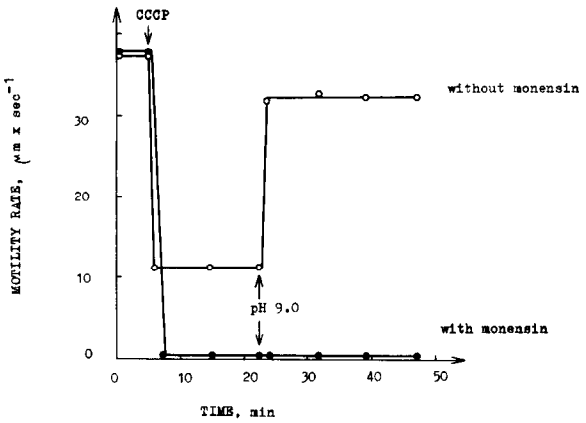

Fig.1. Motility of *V. alginolyticus* at neutral and alkaline pH. Bacteria were incubated in growth medium 2. Alkalinization to pH 9.0 was achieved by adding Tris. Other additions, 2×10^{-5} M CCCP and 3×10^{-5} M monensin.

Fig.3 shows the Na^+-dependence of *V. alginolyticus* motility. It is seen that for motility in the presence of CCCP, it is necessary to have high Na^+ outside the cell. The 6–12-fold decrease in the extracellular Na^+ level arrest motility. The same decrease was almost without effect if CCCP was omitted. (Decrease in external Na^+ below 30 mM proved to be impossibe because of lysis of the cells.) In these experiments, NaCl could not be substituted by KCl (not shown). Results of the inhibitor analysis of *V. alginolyticus* motility are given in table 1. From the obtained data, one can conclude that there are at least two different

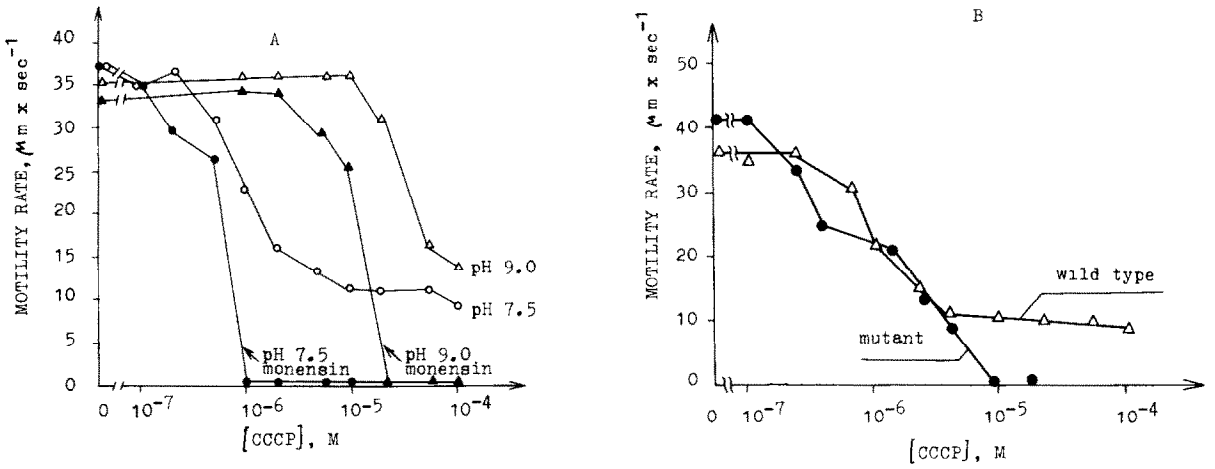

Fig.2. CCCP titration of the motility rate of *V. alginolyticus* wild strain (A) and mutant growing on acetate (B). Addition: 3×10^{-5} M monensin. Conditions as in fig.1.

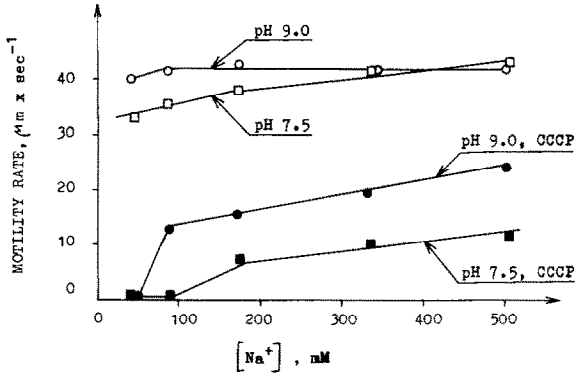

Fig.3. Na^+ dependence of *V. alginolyticus* motility at pH 7.5 and 9.0 with or without 2×10^{-5} M CCCP. Conditions as in fig.1.

Table 1

Inhibitor analysis of the *V. alginolyticus* motility

pH	Additions	Motility rate ($\mu m \times s^{-1}$)
9.0	–	36.0
	KCN	14.5
	KCN + vanadate	9.5
	KCN + arsenate	0
	KCN + CCCP	12.0
	KCN + CCCP + vanadate	0
7.5	–	35.5
	CCCP	12.0
	KCN + CCCP	10.0
	KCN + CCCP + vanadate	0

Additions: 2×10^{-4} M KCN, 2×10^{-4} M vanadate, 1×10^{-2} M arsenate, 2×10^{-5} M CCCP

mechanisms supplying energy for the flagellar motor of these bacteria. The mechanisms in question are specifically inhibited by (i) cyanide, (ii) arsenate or vanadate + CCCP. Instead of KCN, anaerobiosis can be used (not shown).

In the last series of experiments, an attempt was undertaken to select a mutant lacking Na^+-linked energy coupling. The strategy of this selection was the following. According to [7], all the Na^+-solute symporters have a common protein subunit. One may speculate that such a subunit is a constituent of the $\Delta\bar{\mu}_{Na}$-driven flagellar motor. If this were the case it would be hoped that a mutant deficient in two different Na^+-solute symports should be also defective in $\Delta\bar{\mu}_{Na}$-generating systems and/or $\Delta\bar{\mu}_{Na}$-driven motility.

A strain unable to utilize added glycerol and threonine has been isolated. It was revealed that it could not grow on tryptone, indicating that not only threonine but also other amino acids imported with Na^+ [5] could not be utilized by the cells. On

the other hand, the strain could grow on acetate easily penetrating biomembranes without carriers in its protonated form. In agreement with the above reasoning, the motility of such a mutant was completely arrested by CCCP without monensin at pH 7.5 (fig.2b) as well as at pH 9.0 (not shown).

4. DISCUSSION

The above data seem to be sufficient to conclude that motility of *V. alginolyticus* can be supported by operation of a flagellar motor driven by $\Delta\bar{\mu}_{Na}$.

(i) Motility can be observed in the presence of a large amount of protonophorous uncoupler CCCP. Such an effect cannot be explained by the ineffectiveness of CCCP under the conditions used since in the presence of monensin, much lower concentrations of CCCP completely arrested motility (fig.1,2).

(ii) In the CCCP-containing media, an Na^+ gradient (low $[Na^+]_{in}$ and high $[Na^+]_{out}$) is necessary for motility. In fact, lowering $[Na^+]_{out}$ paralysed the cells in the presence, but not in the absence of CCCP (fig.3). On the other hand, addition of monensin to samples with CCCP immediately stopped motility. Under these conditions, monensin was found to increase $[Na]_{in}$ as was demonstrated in this group by Dr I.A. Smirnova using the NMR method (not shown).

The effect of monensin in this system can be easily explained if we assume, as in [5], that in *V. alginolyticus* there are $\Delta\bar{\mu}_{Na}$-generator(s), say an Na^+-motive respiratory chain. In the absence of CCCP, the produced $\Delta\bar{\mu}_{Na}$ is composed of $\Delta\Psi$ (inside negative) and ΔpNa ($[Na^+]_{out} > [Na^+]_{in}$). The proton-conducting pathway organized by CCCP induced the H^+ influx down $\Delta\Psi$ so that the constituent of $\Delta\bar{\mu}_{Na}$ converts into ΔpNa. Under these conditions, monensin must abolish ΔpNa, exchanging extracellular Na^+ for intracellular H^+. As a result, both components of $\Delta\bar{\mu}_{Na}$ collapse.

In the literature there are two indications that alkalophilic bacteria possess $\Delta\bar{\mu}_{Na}$-driven motors. Hirota et al. [8] reported that motility of an alkalophilic *Bacillus* at pH 9.0 (i) requires Na^+, (ii) is arrested by valinomycin + K^+ or nigericin and (iii) decreased down to 45 and 27% of the initial rate by CCCP and monensin, respectively. The authors entitled their paper

'Flagellar motors of alkalophilic *Bacillus* are powered by $\Delta\bar{\mu}_{Na}$'

Unfortunately, an alternative explanation of these data is not excluded. In alkalophiles, Na^+ is known to be necessary to maintain neutral intracellar pH. If an electrogenic Na^+/nH^+ antiporter is involved [9] addition of electroneutral Na^+/H^+ antiporting nigericin and monensin should induce alkalinization of the cytoplasm. Valinomycin should do the same converting $\Delta\Psi$ to ΔpH (interior alkaline). So, in all the cases mentioned, inhibition of motility could be simply a result of $[H^+]_{in}$ decrease down to a non-physiological level. The same explanation may be valid for the results by Kitada et al. [10]. The authors found that motility of alkalophilic *Bacillus firmus* is activated by including 10 mM NaCl in an Na^+-free incubation mixture. Under the same conditions, $\Delta\Psi$ was no more than slightly elevated by NaCl. Again, valinomycin + K^+ completely paralysed the cells.

To avoid the above-mentioned alternative, we measured motility of *V. alginolyticus* not only at pH 9.0 but also at pH 7.5. It proved to be possible since this microorganism, in contrast to *Bacillus firmus*, is motile in both alkaline and neutral media. At pH 7.5 and in the presence of CCCP, an increase of the intracellular Na^+ level by monensin or a decrease of $[Na^+]_{out}$ by lowering NaCl in the incubation mixture were found to make bacteria motionless. In the absence of CCCP, monensin addition and $[Na^+]_{out}$ decrease were without such a dramatic effect. These relationships show that the role of Na^+ is really to power the flagellar motor rather than to prevent cytoplasmic alkalinization, or to maintain intact either the motor or some other constituents of the cell.

Data of the inhibitor analysis (table 1) suggest that besides the Na^+-motive respiratory chain, *V. alginolyticus* possesses one more $\Delta\bar{\mu}_{Na}$-generating mechanism which is sensitive to vanadate and arsenate and resistant to cyanide and anaerobiosis. Motility that utilizes energy produced by this mechanism ceases when $[Na^+]_{out}$ is lowered or monensin added to increase $[Na^+]_{in}$. As found in this group, there is a DCCD-resistant ATPase in *V. alginolyticus* subbacterial particles, which is inhibited by vanadate [11,12].

The activity proved to be identical to Cl^--activated nucleotidase earlier described in this bacterium by Hayashi et al. [13]. The hydrolysis was activated 12-fold by Cl^- (half-maximal effect at 0.6 mM chloride). VO_4^{3-} completely abolished the effect of Cl^-. Such a system, however, is hardly responsible for the effect of vanadate on motility since this motility could be arrested by monensin. The question of Na^+-activated ATPase sensitive to vanadate is now under investigation.

It should be noted in this connection that experiments on intact cells performed here by Dr M.Yu. Galperin revealed a vanadate induced decrease in the aerobic level of intracellular ATP. A possible explanation of this fact is that $\Delta\bar{\mu}_{Na}$ generated by the Na^+-motive respiratory chain can reverse a vanadate-sensitive Na^+-motive ATPase which, under conditions of active respiration, forms ATP.

Combining the above data with those of Tokuda et al. [5, 6, 14], one may construct the following scheme of the Na^+ cycle that is functionally similar to the H^+ cycle found in neutro- and acidophilic organisms. According to fig. 4, $\Delta\bar{\mu}_{Na}$ is produced by an Na^+-motive respiratory chain and is utilized by Na^+-solute symporters, the flagellar $\Delta\bar{\mu}_{Na}$-driven motor and maybe Na^+-ATP-synthase to perform osmotic, mechanical and chemical work, respectively.

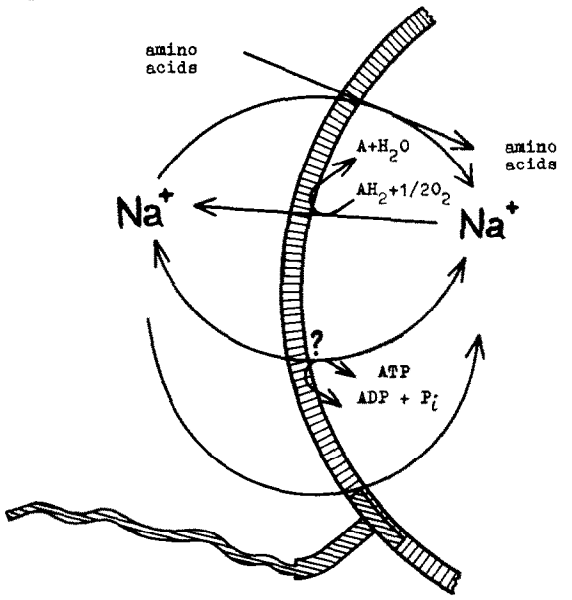

Fig.4. Tentative scheme of the Na^+-linked energetics in *V. alginolyticus*.

An alternative scheme may include two coupling membranes, the outer bearing $\Delta\bar{\mu}_{Na}$ and specialized in performance of osmotic (transport) and mechanical (motility) work, and the inner bearing $\Delta\bar{\mu}_H$ and catalyzing oxidative phosphorylation of the usual type.

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