Ion Selectivity of the *Vibrio alginolyticus* Flagellar Motor

JENNY Z. LIU, MICHAELA DAPICE, AND SHAHID KHAN*

Departments of Anatomy and Structural Biology, Physiology, and Biophysics, Albert Einstein
College of Medicine, Bronx, New York 10461

Received 27 February 1990/Accepted 31 May 1990

The marine bacterium, *Vibrio alginolyticus*, normally requires sodium for motility. We found that lithium will substitute for sodium. In neutral pH buffers, the membrane potential and swimming speed of glycolyzing bacteria reached maximal values as sodium or lithium concentration was increased. While the maximal potentials obtained in the two cations were comparable, the maximal swimming speed was substantially lower in lithium. Over a wide range of sodium concentration, the bacteria maintained an invariant sodium electrochemical potential as determined by membrane potential and intracellular sodium measurements. Over this range the increase of swimming speed took Michaelis-Menten form. Artificial energization of swimming motility required imposition of a voltage difference in concert with a sodium pulse. The cation selectivity and concentration dependence exhibited by the motile apparatus depended on the viscosity of the medium. In high-viscosity media, swimming speeds were relatively independent of either ion type or concentration. These facts parallel and extend observations of the swimming behavior of bacteria propelled by proton-powered flagella. In particular, they show that ion transfers limit unloaded motor speed in this bacterium and imply that the coupling between ion transfers and force generation must be fairly tight.

The rotation of bacterial flagella (6) is energized by ion gradients rather than ATP (28). The chemical components and the structural architecture of these molecular motors is being worked out in the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* (32) (reviewed by Macnab and DeRosier [27]). The energetics and dynamics of flagellar rotation have been most extensively characterized in a motile *Streptococcus* species (reviewed by Khan [17a]). These and most bacteria studied thus far require proton gradients for motility. The relationships between motor rotation frequency, torque, and the driving proton potential depend on the pH and the viscous load (19). When cells are tethered onto glass by one flagellum, a single motor powers the rotation of the whole cell. The load is high and the running torque is large (ca. 10^{-11} dyne-cm). During swimming, flagellar motors rotate close to zero external load at high speeds and low (ca. 10^{-13} dyne-cm) torque (25). The role of proton transfers in limiting motor speed has been probed by study of hydrogen isotope effects. Isotope effects were absent during tethered cell rotation (9, 18) but prominent during rapid rotation of flagellar bundles in free-swimming cells (9, 25).

An increasing number of bacterial species are being identified in which flagellar rotation depends on sodium gradients (4, 11, 12, 16, 17, 33, 34). Identification, upon comparison with the proton motors, of the changes in machinery and mechanism that provide the basis for the sodium dependence should yield valuable clues about the coupling between ion transfers and force generation. We began work on *Vibrio alginolyticus* guided by this consideration. We chose *V. alginolyticus* since it (i) has well-characterized physiology (35, 36), (ii) exhibits good motility at neutral pH, at which proton pumps maintain the membrane potential independently of sodium (36), and (iii) swims by means of a single flagellum so that hydrodynamic considerations of bundle formation do not complicate analysis. We found that the *V. alginolyticus* flagellar motor can utilize lithium instead of

sodium. We exploited the selectivity of this cation-powered motor in analogous fashion to the studies of isotope discrimination exhibited by proton-powered flagellar motors. Our findings illuminate the role of ion transfers in limiting motor speed and define a number of characteristics of the ion transporters coupled to motility.

MATERIALS AND METHODS

Materials. Lithium chloride was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.) (>99.999% pure) and Sigma Chemical Co. (St. Louis, Mo.) (>99.9% pure). The ionophores monensin (Sigma) and valinomycin (Sigma) were stocked as 10- and 1-mg/ml methanolic solutions, respectively. Silicone oil (Aldrich) and *n*-octane (Sigma) were used as a 90:10 mixture. [^3H]tetraphenylphosphonium ([^3H]TTPP), $^3\text{H}_2\text{O}$, and [^{14}C]inulin were obtained from Amersham Corp. (Arlington Heights, Ill.). ^{86}Rb and the scintillant Aquasol-2 were from Dupont, NEN Research Products (Boston, Mass.).

Cell growth and preparation. *V. alginolyticus* 4109 cells were inoculated from motility rings from 0.3% soft agar plates (1) into a salt medium (0.3 M NaCl, 10 mM KCl, 2 mM K_2HPO_4 , 15 mM MgSO_4 , 50 mM Tris hydrochloride, pH 7.5) supplemented with 0.5% peptone and 0.1% yeast extract. The cells were grown aerobically at 37°C on a shaking water bath and harvested at the middle to late log phase. The harvested cells were washed once in 0.4 M KCl–50 mM Tris hydrochloride (pH 7.5) and then K^+ loaded by incubation at room temperature for 15 min with 0.4 M KCl–50 mM diethanolamine hydrochloride (pH 8.5) (36). After washing and a further 15 min of incubation with 0.4 M KCl–50 mM diethanolamine hydrochloride (pH 8.5), the cells were washed twice with 0.4 M KCl–50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.0), resuspended in the same washing buffer at pH 7.0, and kept on ice until use (<1 h). For loading with cesium, 0.4 M KCl in the diethanolamine hydrochloride (pH 8.5) buffer was replaced with 0.4 M CsCl. For ionophore studies, cells were EDTA treated by suspension in 0.1 M Tris hydrochloride (pH 8.1)–0.4 M KCl–10 mM K^+ -EDTA just after loading.

* Corresponding author.

Motility measurements. Swimming speed of the cells was monitored in 50 mM HEPES buffers at pH 7.0 containing 0.2% glucose and 0.4 M salts with various compositions. Measurements were made at room temperature, using cross-bridged cover slips. In experiments involving increased viscous loads, Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, N.J.) (21) was added to the media from concentrated stock solutions. Kinematic viscosities were measured with a Cannon-Ubbelohde viscometer (size 100). Experiments were videotaped, and swimming speeds were determined manually or by computer as described previously (19).

Membrane potential and intracellular sodium determinations. [^3H]TPP (specific activity, 26 Ci/mmol; final activity, 3 $\mu\text{Ci/ml}$) and 0.2% glucose were added to the cell suspension. Duplicate 0.2-ml samples were withdrawn at periodic intervals and centrifuged through a 90:10 mixture of silicone oil-*n*-octane. A 0.1-ml sample of the supernatant was withdrawn for counting, and the rest, together with the oil, was removed by suction. At the end of the experiment, 5% butanol was added and a sample pair was collected for estimation of the background. The pellets were processed and the radioactivity was assessed as described previously (19). In double-label experiments on EDTA-valinomycin (2 $\mu\text{g/ml}$, final concentration)-treated cells, [^3H]TPP gave values ca. -20 mV higher than ^{86}Rb (specific activity, 400 mCi/mmol; final activity, 0.1 $\mu\text{Ci/ml}$), but relative changes in potential registered by the probes as a function of K^+ concentration were the same. Internal cell water in the pellets obtained in these experiments upon centrifugation through silicone oil-*n*-octane was estimated by the combined use of $^3\text{H}_2\text{O}$ and [^{14}C]inulin as detailed by Khan et al. (19). From these measurements, we determined that 0.75 μl of internal cell water per ml of cell suspension corresponded to 9.7×10^9 cells per ml, or 1 optical density unit at 600 nm as read in a Bausch & Lomb Spectronic 20 spectrophotometer. From the cation uptake, the membrane potential, $\Delta\psi$, was determined from the Nernst equation, $\Delta\psi = -(2.3RT/F) \log [\text{TPP}_{\text{in}}/\text{TPP}_{\text{out}}]$, where F is faradays, R is the gas constant, and T is the temperature.

$^{22}\text{NaCl}$ (specific activity, 16.4 Ci/mmol) was used for measurement of internal Na^+ concentration. $^{22}\text{NaCl}$ (1 $\mu\text{Ci/ml}$ in 5 M NaCl stock solution) was used to make up 50 mM HEPES (pH 7.0)-0.4 M NaCl buffer. The Na^+ composition was varied by mixing with 50 mM HEPES (pH 7.0)-0.4 M KCl buffer in the desired ratio. [^{14}C]inulin was added for estimation of the excluded pellet water. Pairs of samples were withdrawn at various times and centrifuged. For each sample, 40 μl of the supernatant was gamma counted (1282 Compugamma Universal) for estimation of the ^{22}Na and another 40 μl was withdrawn to a scintillation vial for estimation of the [^{14}C]inulin. The residual supernatant together with the oil was removed by suction. The Eppendorf tubes containing the pellets were dried and gamma counted for the ^{22}Na , and then their bottoms were clipped and dropped into scintillation vials for estimation of the [^{14}C]inulin.

Artificial energization. K^+ -loaded cells were treated with 0.2 mM KCN and 2 mM arsenate at pH 7.5 as described previously (12), the KCN and arsenate being present in all buffers subsequent to treatment. The cells were divided into two batches. One batch was used to monitor the effect of the inhibitors. The other batch was used for artificial energization of motility or [α - ^{14}C]aminoisobutyric acid uptake. The time required for deenergization effected by the inhibitors at these concentrations was about 30 min. This time was

TABLE 1. Ion dependence of *V. alginolyticus* swimming motility^a

Salt tested	Cell prepn	Motility
NaCl	1, 2	++, ++
NaCH_3COO	1	++
LiCl	1, 2	+, +
KCl	1, 2	-, -
$\text{KCl}_{6.0}$	1, 2	-, -
RbCl	1	-
CsCl	1	-
TiCH_3COO	1	-
NH_4Cl	1	-
NH_3OHCl	1	-

^a The salts were used at 0.4 M concentration in 50 mM HEPES (pH 7.0)-0.2% glucose buffers. Cells were loaded as described in Materials and Methods. 1, K^+ -loaded cells; 2, Cs^+ -loaded cells. For each experiment, the loaded cells were washed twice in buffer containing the salt to be tested. Without sodium and lithium, cells were immotile after the second wash. $\text{KCl}_{6.0}$ catalogs results of experiments in which the dependence of protons on motility was investigated (see text). ++, Vigorous swimming motility; +, slow swimming motility; -, immotility.

determined by suspending cells at various times in sodium buffer (50 mM HEPES [pH 7.5], 0.4 M NaCl) plus 0.2% glucose and monitoring the progressive loss of motility. For artificial energization, the cells were incubated with the inhibitors for this period in potassium buffer (50 mM HEPES [pH 7.5], 0.4 M KCl) and then diluted 1/20 in sodium buffer. Motility was observed and samples were withdrawn for estimation of [α - ^{14}C]aminoisobutyrate (specific activity, 59 Ci/mmol; final concentration, 8 μM) uptake by centrifugation through silicone oil as described above.

Electron microscopy. Cells were negatively stained with uranyl acetate and viewed on 200-mesh grids at 80 kV accelerating voltage in a JEOLCO 100CX electron microscope as previously described (20).

RESULTS

Ion dependence of *V. alginolyticus* flagellar motor. The motility of *V. alginolyticus* cells in buffers containing chloride salts of the alkali metal ions sodium (Na^+), lithium (Li^+), potassium (K^+), rubidium (Rb^+), and cesium (Cs^+) was examined. In addition to alkali metal cations, we tried cations with high sodium channel permeabilities: thallium (Tl^+), ammonium (NH_4^+), and hydroxylamine (NH_3OH^+) (14).

Our results are summarized in Table 1. Motility was only observed in buffers containing either sodium or lithium. We used Cs^+ -loaded as well as K^+ -loaded cells to study the dependence of motility on potassium and protons. In Cs^+ -loaded cells, the K^+ electrochemical potential obtained in KCl was comparable to the Na^+ electrochemical potential obtained in NaCl (35), yet no motility was observed in the KCl buffer (Table 1). In either Cs^+ - or K^+ -loaded cells, motility was not observed when the pH of the 0.4 M KCl-50 mM HEPES buffer was shifted from 7.0 to 6.0. Thallium was used as the acetate salt. We checked that buffers containing sodium as either the acetate or chloride salt gave comparable motility. The failure to obtain motility in thallium and hydroxylamine may have been due to toxic effects on the pumps responsible for generation of the membrane potential. Cells swam slower in lithium than in sodium, but a similar motility pattern, consisting of translational runs alternating with sharp reversals, was obtained in both cases. In rare instances, cells spontaneously tethered to glass by their

FIG. 1. Concentration dependence of the sodium potential in glycolyzing *V. alginolyticus*. Top: Membrane potential. Each point represents the mean potential, as estimated by [^3H]TPP uptake, of triplicate determinations on a single culture. Bottom: Intracellular sodium and sodium chemical potential. Intracellular sodium was determined by ^{23}Na uptake. Each point represents the mean of triplicate determinations on a single culture. The sodium chemical potential, $\Delta\mu_{\text{Na}}$, was calculated from the measured internal (Na_i) and known external (Na_o) concentrations: $\Delta\mu_{\text{Na}} = (2.3RT/F) \log [\text{Na}_i/\text{Na}_o]$. Bars denote SEs.

flagella were observed. These rotated alternately clockwise and counterclockwise (see Fig. 4 in reference 17a). *V. alginolyticus* flagella are sheathed, but under our experimental conditions, as assessed by negative-stain electron microscopy, the sheaths were degraded, possibly as a result of autolysis (13), exposing areas of filament. Thus, the tethered cell rotation resulted, most likely, from attachment of the exposed regions of the flagellar filament to the cover glass.

Sodium concentration dependence of *V. alginolyticus* flagellar motor. The swimming speed and bioenergetic parameters of potassium (K^+)-loaded, glycolyzing *V. alginolyticus* cells were studied at neutral pH (7.0). Figure 1 shows the dependence of the membrane potential, intracellular sodium, and transmembrane sodium gradient on the buffer sodium concentration. The cells maintained a membrane potential of about -110 mV, as measured by [^3H]TPP, which is independent of the buffer sodium down to 20 mM. Even in the absence of sodium, the cells maintained a substantial membrane potential (about -70 mV). These observations confirmed previous measurements made by Tokuda and Umemoto (36) and showed that at neutral pH, the membrane potential in glycolyzing *V. alginolyticus* is maintained predominantly by proton pumps.

Intracellular sodium increased linearly with external sodium, so that the chemical potential $\{(2.3RT/F) \log [\text{Na}_{\text{out}}^+/\text{Na}_{\text{in}}^+]\}$ changed little over the range 2 to 400 mM. ^{23}Na nuclear magnetic resonance measurements in whole cells (10) and ^{23}Na measurements in vesicles (30) of *E. coli* have shown that in this enteric bacterium intracellular sodium is not regulated and varies proportionately with extracellular sodium under constant membrane potential. It is of interest that a similar situation exists for *V. alginolyticus*, a marine bacterium. The lack of intracellular sodium control and its implications for pH homeostasis in bacteria have been considered (26).

The sodium concentration dependence of swimming speed followed Michaelis-Menten form (Fig. 2). The TPP $^+$ and ^{23}Na measurements together showed that over the extracellular sodium range 20 to 400 mM, the sodium electrochemical potential was invariant. No systematic variation was observed, and the values obtained were scattered within 5% of the mean value (-161 mV) over the 20 to 400 mM concentration range (Fig. 2, inset). In contrast, there was a systematic increase ($>30\%$) in swimming speed over this range.

We repeated the experiments of Dibrov et al. (12) which

FIG. 2. Sodium concentration dependence of *V. alginolyticus* swimming speed. Data from two independent experiments, denoted by different symbols, are shown. Each data point represents a population mean of ca. 30 cells. Bars denote SEs. Inset: Sodium electrochemical potential as a function of sodium concentration. Concentration in millimolars is plotted on the abscissa. Electrochemical potentials were obtained by summation of the membrane and chemical potentials (Fig. 1). They were normalized by division with the mean electrochemical potential obtained over the 20 to 400 mM concentration range. The normalized potentials were plotted on the ordinate.

established sodium energization of the *V. alginolyticus* flagellar motor. We confirmed that the sodium potential-collapsing ionophore, monensin, will decrease swimming speed without affecting the membrane potential. Upon the addition of 50 μ M monensin to EDTA-valinomycin-treated cells, the swimming speed decreased ca. 30% from 25.6 to 17.6 μ m/s (population means of 30 to 50 cells) within 1 to 2 min after addition of the ionophore, while the membrane potential remained unchanged (-91 to -95 mV). We could not confirm transient energization of motility in KCN-arsenate-poisoned, EDTA-valinomycin-treated *V. alginolyticus* cells upon application of a sodium pulse alone, as reported by Dibrov et al. (12). In three independent experiments, we could always energize α -aminoisobutyrate uptake (35), but never motility. However, when a potassium diffusion potential was applied in concert with a sodium pulse, motility was transiently restored in the poisoned cells. It may be that a residual membrane potential was present in the cells used by Dibrov et al. (12). Alternatively, while we have, to the best of our knowledge, copied their published protocol, there may be differences in unspecified parameters between our two protocols.

Ion selectivity of *V. alginolyticus* flagellar motor. The membrane potential and swimming speed were measured as a function of lithium concentration and compared with the measurements obtained in sodium (Fig. 3). These measurements indicated that the motile apparatus discriminates strongly between lithium and sodium. Beyond 100 mM lithium, glycolyzing *V. alginolyticus* maintained a membrane potential which was indistinguishable within error from the

potentials obtained in sodium buffers. In contrast, saturation swimming speeds obtained for the two cases were markedly different. The concentration dependence of the initial rise in swimming speed in the two cases could not be interpreted meaningfully owing to the associated changes in the membrane potential (Fig. 3, top). Beyond 2 mM sodium or 40 mM lithium, the membrane potential obtained was indistinguishable within error from the potential generated in potassium buffers alone.

Swimming speed saturated at lithium concentrations beyond 80 mM. Study of cells grown in high salt confirmed the swimming speed saturation observed in lithium. Cells were grown in 1 M growth medium and loaded with 1 M KCl. Their swimming speeds were observed over a range of LiCl from 0.4 to 1 M, with KCl being used to maintain the 1 M ionic strength. Mean speeds of about 7.57 (standard error [SE] = 0.72) μ m/s, similar to saturation speeds in buffers of standard ionic strength, were obtained over the entire range independent of concentration. These observations, in addition, ruled out the possibility that the motility observed in lithium buffers was due to sodium contamination of the lithium salts used. Were this the case, swimming speed would have continued to increase with increasing lithium salt concentration since a three- to fourfold-higher saturation speed was obtained in sodium buffers.

We tested whether alkalophilic *Bacillus firmus* RAB would swim in sodium-free lithium buffers. We followed protocols used for other alkalophilic *Bacillus* strains (16). Consistent with results obtained on the other strains, swimming motility was not observed. The explanation for the

FIG. 3. Ion selectivity of the *V. alginolyticus* flagellar motor. Open symbols, Sodium. Closed symbols, Lithium. Top: Membrane potential. The sodium data of Fig. 1, top, are reproduced. The membrane potential in lithium was determined as detailed in the legend to Fig. 1 for sodium. Each data point represents an independent experiment with associated SE. Bottom: Swimming speed. The sodium data of Fig. 2 are reproduced. In lithium, cells were immotile at 20 mM and below. Different symbols denote data from separate experiments. Each data point represents a population mean of ca. 30 cells with associated SE. The solid lines represent simulated fits to the data by the zero-load speed equation given in the legend to Fig. 5. Y. Imae (personal communication) has also found that *V. alginolyticus* will swim in lithium.

apparently discrepant behavior between the sodium-driven motility of *B. firmus* RAB and *V. alginolyticus* could lie, in part, in the different flagellation. The slower motor rotation frequencies obtained in lithium may not be sufficient for formation of a stable flagellar bundle in the multiply flagellated RAB. It is also possible that there was significant retention of intracellular sodium in lithium buffers since the intracellular ionic composition could not be controlled by loading as was done for *V. alginolyticus*. Flagellar motors are reversible devices (18). Thus, if sodium was retained, the flux resulting from the outwardly directed sodium gradient could have overwhelmed the inward lithium flux in the RAB motors. Finally, we determined from motility inhibition plots as in reference 33 that the amiloride sensitivity of the *V. alginolyticus* flagellar motor was similar (K_i of ca. 36 μ M) to that reported for *B. firmus* RAB (K_i of ca. 200 μ M) (33) (data not shown).

Modulation of cation selectivity and concentration dependence of *V. alginolyticus* motor speed by viscous load. A fundamental property of a tightly coupled motor is that the

stall torque is proportional to the energizing ion potential, independent of ion type or concentration (19, 23, 29). The radiolabeled measurements indicated that upon decrease of sodium concentration or upon substitution with lithium, the driving ion electrochemical potential changed little. At 5 and 400 mM buffer sodium, the sodium electrochemical potential was 134.5 (SE = 15.1) and 158 (SE = 21.4) mV, respectively. Between 100 and 400 mM, the mean membrane potential was 110 (SE = 6) and 99.7 (SE = 10) mV in sodium and lithium, respectively; it is reasonable to expect that intracellular lithium would vary with extracellular concentration changes in a manner similar to sodium. We asked whether the concentration dependence and ion selectivity of the *V. alginolyticus* flagellar motor decreased as the running torque was brought near the stall torque by increasing viscous load.

The changes in swimming speed obtained when the viscosity of the medium was increased by Ficoll are shown in Fig. 4. In one experiment, changes in swimming speed in 5 and 400 mM sodium buffers were compared. The ratio of swimming speed at the two concentrations changed from 5.4

FIG. 4. Viscosity dependence of *V. alginolyticus* swimming speed. Top: Symbols: ○, 400 mM sodium; ●, 5 mM sodium. Bottom: Symbols: ○, 400 mM sodium; ●, 400 mM lithium. Measurements were made on a single culture for each panel. Each data point represents a population mean of ca. 20 to 30 cells with associated SE.

(400 mM/5 mM speeds) in aqueous buffer (0.85 cP) to 1.275 in 30.8-cP Ficoll buffers. In another experiment, changes in swimming speed in 400 mM lithium and sodium were compared. In this case, the ratio of swimming speed changed from 2.5 (sodium/lithium speeds) in aqueous buffer to 1.5 in 32.6-cP Ficoll buffers. The experiments were duplicated. In every experiment performed, discrimination between ion type or concentration decreased as load was increased.

DISCUSSION

The case for sodium-powered motility in *V. alginolyticus* and other bacteria rests on the following facts. (i) The bacteria are immotile when sodium is removed from the external medium, even though electrical and ion gradients are maintained across the cell membrane (11, 17). (ii) A proton potential does not drive motility in these bacteria. Cells remain motile when the proton potential is collapsed by protonophores such as carbonyl cyanide *m*-chlorophenylhydrazone (11). pH jumps in poisoned bacteria do not restore motility (12). (iii) Changes in swimming speed reflect changes in the transmembrane sodium concentration gradient rather than changes in extracellular or intracellular

sodium since sodium gradient-collapsing ionophores such as monensin specifically affect motility (12, 16; this study). In certain cases, equivalent increases in the membrane potential or the sodium chemical potential produce equivalent increases in swimming speed (16). (iv) Inhibitors of sodium transport systems, namely, amiloride and its analogs (e.g., phenamil), strongly inhibit motility at concentrations which do not affect membrane potential in these bacteria (33) or motility in proton-propelled bacteria (4). (v) Motility in poisoned *V. alginolyticus* cells may be restored by application of a sodium pulse (12).

Thus, the case for sodium-powered motility is good. However, numerous mechanistic questions remain. In particular, the following questions may be asked. (i) Is the sodium flux directly coupled to force generation as often assumed (e.g., reference 7) or are other energy gradients or intermediates involved? (ii) Is coupling tight or loose? (iii) What is the nature of the events that limit unloaded motor rotation? (iv) What are the characteristics of the sodium-transporting pathways?

The findings of the present study may be rationalized, and the questions raised above discussed, within the framework

FIG. 5. Ion transfer reactions linked to flagellar rotation. The motor is composed of several independent functional units (8). Within each unit ions are transferred single file; each ion transfer is linked to a fixed angular displacement of the rotor relative to the stator. Energy coupling occurs over a short spatial distance (energy-coupling site). Ions binding to saturable groups predicate force-generating mechanical transitions which in turn allow ion dissociation and release into the cytoplasm. Force generation is powered by the local ion activity gradient obtained over this distance. The reactions may be decomposed into ion transfers to and from the energy-coupling site (a) and the mechanical transitions (b), joined together in series as schematized. ΔE is free energy. X_{ext} and X_{cyt} denote bulk-phase activities of the energizing ions in the exterior and the cytoplasm, respectively. X_o and X_i denote local activities obtained after the profile of the electric field has been crossed by the ions from the exterior and the cytoplasm, respectively (ions from the exterior alone cross the field in the example schematized). The ion transfer reactions may be decomposed into ion movements across the membrane electric field (a_i) and ion movements independent of the electric field (a_{ii}). The load will affect mechanical (barrier [b]) transitions by changing reactant ground-state free energies, as shown by the solid arrows ($\rightarrow$). The membrane potential, $\Delta\psi$, will affect transmembrane ion (barrier [a_i]) transfers by changing activation as well as reactant ground-state free energies, as shown by the dotted arrows ($\cdots\rightarrow$). When the a_i transfers are rate limiting, the well approximation (18) will not be valid and the electrical and chemical potentials will not be kinetically equivalent. At the high membrane potentials obtained in glycolyzing bacteria, transmembrane ion transfers will not limit rate. Then, zero-load speed = $k_b(X_o - X_i)/2\{(K_d' + X_o)(K_d' + X_i)/K_d' + (k_b/k_a K_d')(2K_d' + X_o + X_i)\}$, as derived in reference 19. The apparent affinity $K_d' = K_d \exp(-e\Delta\psi d/kT)$, where e is unit electron charge, k is the Boltzmann constant, K_d is the dissociation constant, and d is the electrical distance. k_b is the rate constant for the mechanical transitions, and k_a is the bimolecular rate constant for voltage-independent ion transfers, assumed to be the same for transfers from either exterior or cytoplasm to the energy-coupling site.

of a kinetic scheme for transmembrane transit of the energizing ions (Fig. 5). As explained in reference 19, the scheme considers a minimal set of reactions, incorporating simplifying assumptions which have been commonly used in the development of motor models (7, 23, 29). The energetics of this scheme have been analyzed (19). A qualitative account (Fig. 5, legend) will suffice here.

(i) Is coupling direct or indirect? Our results are consistent with those obtained in earlier studies (11, 12) as regards the action of the sodium gradient-collapsing ionophore, monensin. However, we were unable to energize motility by sodium concentration jumps alone. At high pH, starved streptococci rotate when tethered but do not swim when subjected to a pH jump alone. Upon imposition of an electrical potential, swimming was observed in the starved cells (19). The observations on *Streptococcus* species may be explained by noting that transit of the energizing protons will be facilitated by voltage at the light loads encountered during swimming, since transmembrane transport is rate limiting, but not at the high loads encountered during tethered cell rotation, since the mechanical transitions governing force generation are now rate limiting (Fig. 5). A threshold rate of flagellar rotation might be required for cell swimming. Our observations on artificial energization of swimming motility in poisoned *V. alginolyticus* are consistent with a similar situation obtaining in these bacteria. While this interpretation, which we favor, needs to be tested by sodium pulse energization of tethered bacteria, the flagellar sheath makes routine tethering of *V. alginolyticus* unfeasible. Thus, for the present, alternative possibilities cannot be excluded. In particular, coupling might be indirect, with other electrochemical gradients being necessary. The energizing sodium

flux might symport with protons, for example. This possibility also applies to the alkalophilic bacilli and other sodium-powered bacteria, since artificial energization has not been studied in these species.

(ii) Is coupling tight or loose? When transit of each ion is obligatorily coupled to a fixed angular displacement, coupling is tight. In this case, both rotation rate and ion flux will decrease correspondingly upon increasing viscous load. In terms of the scheme depicted in Fig. 5, the local ion activities, X_o and X_i , will approach equilibrium $[(kT/e) \log (X_o/X_i) = -\Delta\psi + (kT/e) \log (X_{\text{ext}}/X_{\text{int}})]$ and transit rate will be determined by the mechanical transitions (barrier [b]). Given constant electrochemical potential, the stall torque will be independent of ion type or concentration since X_o/X_i will be independent of the nature or absolute values of X_{ext} and X_{int} . The ion selectivity and concentration dependence of motor speed near stall will be small in contrast to the case for unloaded motor speed. When coupling is loose, a small fraction of the energy dissipated by the ion flux is coupled to work production. In this case barrier b (Fig. 5) is leaky owing to the presence of alternative pathways. X_o and X_i will not equilibrate with X_{ext} and X_{cyt} , respectively, changing little upon increase of viscous load. Thus, for a loosely coupled mechanism, the ion selectivity and concentration dependence of flagellar motor speed will be insensitive to changes of external medium viscosity.

Quantitative analysis of the changes in swimming speed observed upon changing viscous load awaits determination of the relationship of *V. alginolyticus* swimming speed to its flagellar motor speed. Nor is the stall torque or zero-load speed of the *V. alginolyticus* flagellar motor presently known. Nevertheless, qualitatively it is clear that the swim-

ming speed-viscosity curves (Fig. 4) converge upon extrapolation to zero swimming speed. This implies that the stall torque of the *V. alginolyticus* flagellar motor is relatively insensitive to changes of ion type and concentration. Thus, independent of whether coupling is direct or indirect, the viscosity dependence of *V. alginolyticus* swimming behavior indicates that its flagellar motor rotation is quite tightly coupled to the energizing ion gradients. In doing so, the viscosity dependence also provides strong additional evidence for the energization of motility by the cations.

(iii) What is the nature of the events limiting motor speed? Previous studies on the motility of *V. alginolyticus* recorded changes in swimming speed as a function of external sodium concentration in metabolizing K^+ -loaded cells (11, 12) and Na^+ -loaded cells (34). However, parallel measurements of membrane potential or intracellular sodium were not made, so that the relative contributions of the bioenergetic parameters (sodium activities, chemical potential, and membrane potential) affecting swimming speed could not be evaluated. Our data (Fig. 2) show that changes in swimming speed are determined by the concentration difference of the driving ion, $X_o - X_i$, rather than its concentration gradient, $-\Delta\Psi + (kT/e) \log (X_o/X_i)$. Under conditions in which the electrochemical potential remains invariant but $X_{ext} - X_{cyt}$ is increased, swimming speed increases. This result implies that during swimming *V. alginolyticus* flagellar motors operate far from the mechanochemical equilibrium obtained upon stall, behavior analogous to that exhibited by the proton-driven *Streptococcus* flagellar motors (19, 25).

The fact that saturation swimming speeds in sodium and lithium are substantially different (Fig. 3) implies that ion transfers limit motor speed. At saturation, ion transits are independent of changes in the transmembrane activities, X_{ext} and X_{cyt} , but would depend on differences in k_a or k_b (Fig. 5). If transit rate were determined entirely by mechanical transitions (i.e., k_b) independent of ion transfers (i.e., k_a), then saturation swimming speeds in the two ions should be the same. The viscosity data (Fig. 4) confirm and add to the Fig. 3 data. Extrapolation of the Fig. 4 data toward zero viscosity indicates that the idle speed of the *V. alginolyticus* flagellar motor in lithium is substantially lower than that in sodium.

(iv) What can be said about the cation transporters coupled to motility? Comparative characterization of sodium-dependent flagellar motors has thus far involved the study of amiloride and its analogs (4, 33). The finding of lithium-powered motility gives, additionally, insight on the selectivity characteristics of the ion transporters coupled to motility. The ionic discrimination exhibited by the motile apparatus of *V. alginolyticus* may be compared with that documented for other sodium transport systems. Voltage-gated sodium channels exhibit little discrimination between sodium and lithium permeabilities (15). Amiloride-sensitive sodium channels in epithelia behave similarly (5; J. L. Bremec, R. A. Bridges, R. A. Frizell, and D. J. Benos, Fed. Proc. 46:1270a, 1987). However, lithium permeability is severalfold slower than sodium permeability in Na^+/H^+ antiporters (2). It is thought that the lowered permeability results from slower dissociation of the lithium ion from the carrier site since the affinity of the Na^+/H^+ exchanger for lithium is 5- to 10-fold higher than for sodium (3, 31). While flux data will be necessary for unambiguous interpretation, the reduction of *V. alginolyticus* saturation swimming speed in lithium is consistent with analogous mechanistic considerations being applicable in the motility case as well. Given that *V. alginolyticus* flagellar motors operate far from mechanochemical equilibrium dur-

ing swimming, the cation concentration dependence of swimming speed was fit by the zero-load speed equation (Fig. 5, legend) for the scheme outlined. The fits (k_b/k_aK_d set to 1) yielded dissociation constants, K_d , of 60 and 12 mM for sodium and lithium, respectively. Should motor speed during swimming differ from zero-load speed, the ionic discrimination would be, if anything, underestimated. Nevertheless, the estimated values compare well with binding affinities determined in other sodium transport systems (2, 22). Further, the selectivity sequence may be characterized as Eisenmann sequence X1 (15). This implies that the ion-binding sites have high field strength and, again, like other sodium transporters translocate the bare ions stripped of their hydration shells. Since a sodium ion has dimensions comparable to those of a hydronium ion, subtle modifications of the ion-transporting pathways might be all that are needed to interconvert between proton- and sodium-driven flagellar motors.

It has been suggested, based on the action of amiloride analogs such as phenamil, that sodium-dependent flagellar motors are structurally more similar to channels than to antiporters (33). However, the apparent amiloride affinities of antiporters and sodium-dependent flagellar motors are comparable, whereas amiloride-sensitive sodium channels have apparent affinities below 1 μ M (22). The dependence of the effectiveness of the drugs on factors such as the sodium activity around, and electrical distance of, the drug-binding sites further confounds the issue. Finally, classification in terms of channels and carriers may not have fundamental significance with regard to energy-transducing devices. Ion translocation through channels (i.e., gated pores) if limited by conformational transitions could exhibit features, such as lowered flux, characteristic of carriers (24). Physiologically important carriers are thought to be pores "closed at one end by a molecular machine that accomplishes the coupled translocation steps over a short distance" (15). Thus, the similarity in ion selectivity between flagellar motors and carriers could underlie conserved chemistry evolved to couple ion binding to various work functions.

This study has led us to conclude that during swimming (i) *V. alginolyticus* flagellar motors operate far from mechanochemical equilibrium; (ii) ion transfers play a role in limiting motor speed; (iii) a threshold voltage may be required to gate the energizing cation flux; (iv) the rate limitation of ion transfer reactions is reduced at high viscous load, implying that coupling is fairly tight. These conclusions strikingly parallel conclusions that we (19) and others (9, 25, 29) have made about the swimming behavior of bacteria with proton-propelled flagella. Further, the ion selectivity suggests that the chemical modifications required for interconversion between the proton and cation transporters powering flagellar rotation are small. Therefore, the study gives strength to the belief (7) that force generation in proton- and cation-powered motors may be based on a common mechanistic strategy.

ACKNOWLEDGMENTS

We thank T. A. Krulwich for the gift of *V. alginolyticus* 4109; Y. Imae for communication of unpublished data; R. Stanley for use of the gamma counter; and A. Finkelstein, T. A. Krulwich, R. M. Macnab, J. Segall, and J. L. Spudich for comments on the manuscript.

This work was supported by Public Health Service grant GM36936 from the National Institutes of Health.

LITERATURE CITED

- Adler, J. 1969. Chemoreceptors in bacteria. *Science* 166:1588-1597.

2. Aronson, P. S. 1985. Kinetic properties of the plasma membrane $\text{Na}^+\text{-H}^+$ exchanger. *Annu. Rev. Physiol.* **47**:545–560.
3. Aronson, P. S., M. A. Suhm, and J. Nee. 1983. Interaction of external H^+ with the $\text{Na}^+\text{-H}^+$ exchanger in renal microvillus membrane vesicles. *J. Biol. Chem.* **258**:6767–6771.
4. Atsumi, T., S. Sugiyama, E. J. Cragoe, Jr., and Y. Imae. 1990. Specific inhibition of the Na^+ -driven flagellar motors of alkalophilic *Bacillus* strains by the amiloride analog phenamil. *J. Bacteriol.* **172**:1634–1639.
5. Benos, D. J., L. J. Mandel, and S. A. Simon. 1980. Cationic selectivity and competition at the sodium entry site in frog skin. *J. Gen. Physiol.* **76**:233–247.
6. Berg, H. C., and R. A. Anderson. 1973. Bacteria swim by rotating their flagellar filaments. *Nature (London)* **245**:380–382.
7. Berg, H. C., and S. Khan. 1983. A model for the flagellar rotary motor, p. 485–497. In H. Sund and C. Veeger (ed.), *Mobility and recognition in cell biology*. De Gruyter, Berlin.
8. Blair, D., and H. C. Berg. 1988. Restoration of torque in defective flagellar motors. *Science* **242**:1678–1681.
9. Blair, D., and H. C. Berg. 1990. The *motA* protein of *Escherichia coli* is a proton conducting component of the flagellar motor. *Cell* **60**:439–449.
10. Castle, A., R. M. Macnab, and R. G. Schulman. 1986. Measurement of intracellular sodium concentration and sodium transport in *Escherichia coli* by ^{23}Na nuclear magnetic resonance. *J. Biol. Chem.* **261**:3288–3294.
11. Chernyak, B. V., P. A. Dibrov, A. N. Glagolev, M. Y. Sherman, and A. N. Skulachev. 1983. A novel type of energetics in a marine alkali-tolerant bacterium. *FEBS Lett.* **164**:38–42.
12. Dibrov, P. A., V. A. Kostyrko, R. I. Lazarova, V. P. Skulachev, and I. A. Smirnova. 1986. The sodium cycle. I. Na^+ -dependent motility and modes of membrane energization in the marine alkalotolerant *Vibrio alginolyticus*. *Biochim. Biophys. Acta* **850**:449–450.
13. Follet, E. A. C., and J. Gordon. 1963. An electron microscope study of *Vibrio* flagella. *J. Gen. Microbiol.* **32**:235–239.
14. Hille, B. 1972. The permeability of the sodium channel to metal cations in myelinated nerve. *J. Gen. Physiol.* **59**:637–658.
15. Hille, B. 1984. Ionic channels of excitable membranes. Sinauer Associates, Inc., Sunderland, Mass.
16. Hirota, N., and Y. Imae. 1983. Na^+ -driven flagellar motors of an alkalophilic *Bacillus* strain YN-1. *J. Biol. Chem.* **258**:10577–10581.
17. Hirota, N., M. Kitada, and Y. Imae. 1981. Flagellar motors of alkalophilic *Bacillus* are powered by an electrochemical potential gradient of Na^+ . *FEBS Lett.* **132**:278–280.
- 17a. Khan, S. 1990. Motility, p. 301–343. In T. A. Krulwich (ed.), *The bacteria: a treatise on structure and function*, vol. 12. Academic Press, Inc., New York.
18. Khan, S., and H. C. Berg. 1983. Isotope and temperature effects in chemiosmotic coupling to the flagellar motor of *Streptococcus*. *Cell* **32**:913–919.
19. Khan, S., M. Dapice, and I. Humayun. 1989. Energy transduction in the bacterial flagellar motor: effects of load and pH. *Biophys. J.* **57**:779–796.
20. Khan, S., M. Dapice, and T. S. Reese. 1988. Effects of *mot* gene expression on the structure of the flagellar motor. *J. Mol. Biol.* **202**:575–584.
21. Khan, S., M. Meister, and H. C. Berg. 1985. Constraints on flagellar rotation. *J. Mol. Biol.* **184**:645–656.
22. Kleymann, T. R., and E. J. Cragoe, Jr. 1988. Amiloride and its analogs as tools in the study of ion transport. *J. Membr. Biol.* **105**:1–21.
23. Lauger, P. 1988. Torque and rotation rate of the bacterial flagellar motor. *Biophys. J.* **53**:53–66.
24. Lauger, P., W. Stephan, and E. Frehland. 1980. Fluctuations of barrier structure in ionic channels. *Biochim. Biophys. Acta* **602**:167–180.
25. Lowe, G., M. Meister, and H. C. Berg. 1987. Rapid rotation of flagellar bundles in swimming bacteria. *Nature (London)* **325**:637–640.
26. Macnab, R. M., and A. Castle. 1987. A variable stoichiometry model for pH homeostasis in bacteria. *Biophys. J.* **52**:637–647.
27. Macnab, R. M., and D. J. DeRosier. 1988. Bacterial flagellar structure and function. *Can. J. Microbiol.* **34**:442–451.
28. Manson, M. D., P. Tedesco, H. C. Berg, and C. van der Drift. 1977. A proton motive force drives bacterial flagella. *Proc. Natl. Acad. Sci. USA* **74**:3060–3064.
29. Meister, M., R. Caplan, and H. C. Berg. 1989. Dynamics of a tightly coupled model for flagellar rotation. *Biophys. J.* **55**:905–914.
30. Reenstra, W. W., L. Patel, H. Rottenberg, and H. R. Kaback. 1980. Electrochemical proton gradient in inverted membrane vesicles from *Escherichia coli*. *Biochemistry* **19**:1–9.
31. Rindler, M. J., and M. H. Saier, Jr. 1979. Uptake of $^{22}\text{Na}^+$ by cultured dog kidney cells (MDCK). *J. Biol. Chem.* **254**:11431–11439.
32. Stallmeyer, M. J. B., S.-I. Aizawa, R. M. Macnab, and D. J. DeRosier. 1988. Image reconstruction of the flagellar basal body of *Salmonella typhimurium*. *J. Mol. Biol.* **205**:519–528.
33. Sugiyama, S., E. Cragoe, Jr., and Y. Imae. 1988. Amiloride, a specific inhibitor for the Na^+ -driven flagellar motor of alkalophilic bacillus. *J. Biol. Chem.* **263**:8215–8219.
34. Tokuda, H., M. Asano, Y. Shimamura, T. Unemoto, S. Sugiyama, and Y. Imae. 1988. Roles of the respiratory Na^+ pump in bioenergetics of *Vibrio alginolyticus*. *Biochem. J.* **103**:650–655.
35. Tokuda, H., M. Sugawara, and T. Unemoto. 1982. Roles of Na^+ and K^+ in α -amino isobutyric acid transport by the marine bacterium *Vibrio alginolyticus*. *J. Biol. Chem.* **257**:788–794.
36. Tokuda, H., and T. Unemoto. 1982. Characterization of the respiration dependent Na^+ pump in the marine bacterium *Vibrio alginolyticus*. *J. Biol. Chem.* **257**:10007–10014.