Acetate acts as a protonophore and differentially affects bead movement and cell migration of the gliding bacterium *Cytophaga johnsonae* (*Flavobacterium johnsoniae*)

JoAnn L. Dzink-Fox, Edward R. Leadbetter and Walter Godchaux, III

Author for correspondence: Edward R. Leadbetter. Tel: +1 860 486 5398. Fax: +1 860 486 1936.
e-mail: erl@uconnvm.uconn.edu

Department of Molecular
and Cell Biology,
University of Connecticut,
Storrs, CT 06269-2131, USA

Cells of *Cytophaga johnsonae* (now *Flavobacterium johnsoniae*) are able to translocate on solid surfaces but are unable to swim in liquid media. Organelles that may be involved in this gliding motility have not been detected, and the mechanism(s) responsible remains unknown. The movement of latex beads attached to the cell surface is considered by some to be a manifestation of the gliding machinery. In this study, acetate (in nutrient-level quantity, 45 mM) was found to inhibit bead movement on cell surfaces, whilst formation and movement of groups of cells (rafts) and typical colony spread were not affected; generation time (in liquid culture) was only slightly increased. Since acetate is a weak acid and is recognized as a protonophore, various electron-transport-associated features were assessed in an effort to understand the differential effects of acetate on bead movement and cell motility. Selected protonophores and electron transport inhibitors were tested to compare their effects on cell translocation and metabolic activities with those of acetate. Although O₂ consumption was not significantly affected in the presence of acetate and the protonmotive force decreased only minimally, ATP levels were markedly decreased. Arsenate and cyanide were also shown to inhibit bead movement but did not inhibit either movement of rafts of cells or colony spreading. Cyanide lowered O₂ consumption, while arsenate did not; both compounds effected substantial decreases in cellular ATP content, but little or no decrease in protonmotive force. The inhibitory effects of these compounds on bead movement over cell surfaces contrasted with the continued ability of cells to form rafts, to glide and to form spreading colonies and led to the conclusion that bead movement is not a complete correlate of the gliding machinery of *C. johnsonae*. In addition, it seems likely that bead movement is more affected by the level of cellular ATP than it is by the protonmotive force, which has been assumed to provide the energy (derived from the transmembrane gradients) for the gliding machinery.

Keywords: bead movement, gliding motility, *Cytophaga johnsonae* (*Flavobacterium johnsoniae*), protonmotive force

INTRODUCTION

The gliding motility of the cytophagas is characterized by the ability of these fusiform Gram-negative cells to

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; PMS, phenazine methosulfate; TPP⁺, tetra[³H]phenylphosphonium bromide.

translocate over a solid surface to form thin colonies with feathery, finger-like edges. Gliding motility requires cell contact with a solid surface or an air–water interface (Weibull, 1960); when placed in a liquid medium, cells are non-motile. To date, no recognizable organelles of motility have been found that might account for cell movement and the mechanisms responsible remain unknown. The sole known molecular correlate of gliding motility is the presence of unusual outer mem-

brane components – sulfonolipids – characteristic of the *Cytophaga-Flexibacter* group (Abbanat *et al.*, 1986; Godchaux & Leadbetter, 1983). These lipids, as well as the ornithine amino lipids, also present in significant quantities (Pitta *et al.*, 1989), are characterized by large amounts of branched-chain fatty acyl moieties. In addition to the cytophagas (Walker, 1968), an unrelated group of gliding bacteria (the myxobacters) also contain especially large amounts of branched-chain fatty acids (Ware & Dworkin, 1973).

Since an increased content of branched-chain fatty acids is considered to increase membrane fluidity and it has long been argued that greater membrane fluidity could be a salient trait of gliding bacteria (Nichols *et al.*, 1986), the large amounts of these compounds may be important for this ability to move over solid surfaces. In one experiment to assess the influence of branched-chain fatty acid content on the ability of *Cytophaga johnsonae* (now *Flavobacterium johnsoniae*) cells to glide, we supplied acetate, in nutrient-level concentrations in a carbon-limited medium in an effort to induce an alteration in the fatty acid composition of membrane lipids from branched- to straight-chain species. Although no significant differences in the profile of cellular fatty acids were found, a trait presumed to be a significant marker of gliding motility – the ability to move latex spheres over cell surfaces – was affected.

Pate & Chang (1979) and Lapidus & Berg (1982) first observed the movement of such microspheres over the surface of gliding bacteria and suggested that it was a manifestation of the gliding machinery presumed present in the cell envelope. This putative relationship of bead movement to gliding ability has however been questioned by us and others because of contradictory observations (Ridgway & Lewin, 1988; Gorski *et al.*, 1991). As the present study reveals, cells grown in or on media containing acetate at concentrations from 35 to 50 mM failed to move attached beads over their surfaces, while colony enlargement (spreading), which is a result of cell migration on the agar surface, was normal.

Acetate, which does not serve as sole source of carbon and energy for growth of *C. johnsonae*, is recognized to dissipate the proton gradient in other bacteria that have been examined. It seemed possible then, that acetate might be acting in a similar manner in this bacterium and so lead to a decrease in the energy supply of the cells. The protonmotive force is regarded as the source of energy for gliding (and, presumably, bead movement) (Duxbury *et al.*, 1980; Pate & Chang, 1979; Ridgway, 1977; Wolkin & Pate, 1986). If the effects of acetate were a result of diminishing the electrochemical gradient of protons across the membrane, the ability of cells to glide (and colonies to spread) could be affected. Accordingly, we examined the effects of acetate and selected inhibitors of electron transport and oxidative phosphorylation on various energy-related activities that might influence gliding of *C. johnsonae*. We report here results of these studies on colony spreading, raft formation and movement, and bead movement, as well as O₂ consumption,

ATP levels, and proton gradient and membrane potential (together constituting the protonmotive force).

METHODS

Bacterial strains and growth conditions. Studies were performed with *C. johnsonae* (ATCC 43786). *Escherichia coli* K-12 was also used for comparison, when appropriate. The growth medium [a modified EC (enriched cytophaga) medium; Pate & Ordal, 1967] contained 3.5 g tryptone and 0.75 g yeast extract per litre, and was adjusted to pH 7.4 before autoclaving; for solid media, 1.8% (w/v) Bacto agar was added. Overnight cultures were grown aerobically to late-exponential phase (OD₆₅₀ 0.8; Cary model 219 spectrophotometer; 1 cm light pathlength) in a waterbath-shaker at 80 r.p.m. at 25 °C (37 °C for *E. coli*) and used as inoculum for each study. Cells were harvested in early- to mid-exponential phase (unless otherwise indicated), depending upon the assay to be performed.

Chemicals. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), dicyclohexylcarbodiimide (DCCD), DNP, valinomycin, nigericin, sodium ascorbate, phenazine methosulfate (PMS), sodium azide, sodium arsenate, luciferin-luciferase assay kit (FL-AAM, FL-AAB) and ATP were from Sigma. Sodium acetate and sodium cyanide were from Fisher Scientific. [7-¹⁴C]Benzoic acid (40 mCi mmol⁻¹, 1.5 GBq mmol⁻¹) and [U-¹⁴C]sucrose (530 mCi mmol⁻¹, 20 GBq mmol⁻¹) were from ICN Biomedicals. Tritiated H₂O was from Amersham; [*carboxyl*-¹⁴C]inulin (0.05 mCi, 1.9 MBq) and tetra[³H]phenylphosphonium bromide (TPP⁺) (37 Ci mmol⁻¹, 1.4 TBq mmol⁻¹) were from Dupont-New England Nuclear.

Colony spreading. *C. johnsonae* was grown to mid-exponential phase (OD₆₅₀ 0.4) and diluted in fresh sterile medium to obtain a few, spatially separated colonies when plated on agar containing a range of concentrations of the various additives (Table 1) and a control without additives. After incubation for up to 3 d, colony type (spreading or not spreading) was noted. The highest concentration of additive that still permitted growth (Table 1) was chosen for additional evaluations of bead and cell movement.

Movement of latex spheres by cells. An overnight culture of *C. johnsonae* was inoculated into fresh broth and incubated until an OD₆₅₀ of 0.1–0.2 was attained. At that time, aliquots of the culture were placed into separate flasks, allowed to equilibrate for a few minutes and the appropriate concentration of inhibitor (Table 1) was then added to each flask. Duplicate samples were removed at designated times to determine optical density and bead movement. A suspension of polystyrene latex beads (0.261 µm; Seragen Diagnostics) was added to the duplicate samples to a density of approximately 50 beads per cell as described by Pate & Chang (1979) and modified by Godchaux *et al.* (1990). Bead movement on the cell surface was immediately assessed in wet mounts for 2–3 min by phase-contrast microscopy at a total magnification of ×400 and scored as either positive or negative. Longer observation times evidently rendered a wet mount anoxic, resulting in the cessation of bead movement on control cells.

Raft formation and movement by cells. The ability of cell populations to form rafts was determined as described previously (Godchaux *et al.*, 1991). For the inhibitor studies, aliquots of liquid cultures (OD₆₅₀ 0.7–0.8) were placed in flasks to which the test agents were added and incubated for various times up to 30 min. Cells were centrifuged and resuspended in non-nutrient buffer (10 mM HEPES, 1 mM MgCl₂, 1 mM CaCl₂) to an approximate OD₆₅₀ of 1.5. It was important to

use a dense suspension of cells to produce large, moving rafts. Droplets (2 μ l) of the cell suspension were then dispensed onto agar-coated slides with or without a test agent, and the rate of raft movement was observed using a phase-contrast microscope ($\times 400$) equipped with an ocular micrometer. Observations were made for up to 10 min. Longer observation times resulted in drying of the agar and cessation of raft movement. Control cells grown in the absence of a test agent were placed onto agar-coated slides both with and without the agents. The rate of raft movement (μ m min⁻¹) in the presence of the agent was expressed as a percentage of the control for each time of exposure to each agent.

Measurement of respiratory activity (O_2 consumption). Overnight cultures were diluted in fresh medium and incubated until an OD_{650} of 0.13–0.2 was obtained. Oxygen consumption was measured at room temperature (approx. 25 °C) with a Clark oxygen electrode (Yellow Springs Instruments) attached to a strip chart recorder. The electrode was calibrated using sodium dithionite to establish an anaerobic base line. After establishing a linear (control) rate of O_2 consumption by a cell suspension, the agent to be tested was introduced into the electrode chamber and O_2 consumption was monitored for 5–10 min. Respiratory rates were calculated as percentages of the control rate.

Measurement of cellular ATP. A culture was inoculated into fresh medium and incubated at 25 °C until an OD_{650} of 0.15–0.2 was attained. (*E. coli* was grown to an OD_{650} of approximately 0.35. These optical densities of *E. coli* and *C. johnsonae* reflect an equivalent number of cells per ml). Aliquots of the culture were placed into flasks, various test agents added and the cultures were incubated at 25 °C for up to 30 min. ATP was extracted from the cells by injecting 125 μ l of cell suspension into 2.5 ml boiling Tris/HCl buffer (0.02 M, pH 7.75) in chromic acid-cleaned screw-capped tubes (Hamilton & Holm-Hansen, 1967; Ridgway, 1977). The samples were boiled for 4–5 min to permit complete extraction of ATP, cooled to room temperature and then frozen at –20 °C until analysis. The ATP content was determined by the luciferase-luciferin assay procedure provided with the Lumat LB9501 Semi-automatic Lumin-ometer (EGG Berthold). ATP levels were calculated by reference to ATP standards and expressed as nmol ATP (mg dry wt cells)⁻¹.

Measurement of intracellular volume. The internal cell volumes of the cytoplasm and periplasm were measured using the methods of Collins & Hamilton (1976) and Stock *et al.* (1977). Overnight cultures were diluted in fresh medium and reincubated until an OD_{650} of approximately 0.4 (equivalent to approx. 0.29 mg dry wt cells ml⁻¹). *E. coli* was grown to an OD_{650} of approximately 0.75 (equivalent to approx. 0.29 mg dry wt cells ml⁻¹). The cells were harvested by centrifugation (12000 g) for 10 min at 4 °C and the cell pellet was resuspended in 10 mM Tris/HCl buffer (pH 7.3) to a density of 10 mg dry wt cells ml⁻¹. Stirred cell suspensions (10 mg dry wt cells ml⁻¹) were incubated with the following radiolabelled compounds for 10 min: [¹⁴C]sucrose/³H₂O (measurement of cytoplasmic volume); [¹⁴C]inulin/³H₂O (measurement of total cell volume). Radioactivities were [¹⁴C]sucrose, 0.05 μ Ci μ l⁻¹ (1.85 kBq μ l⁻¹), sp. act. 530 mCi mmol⁻¹ (19.6 GBq mmol⁻¹); [¹⁴C]inulin, 0.05 μ Ci μ l⁻¹ (1.85 kBq μ l⁻¹), sp. act. 13 mCi mmol⁻¹ (481 MBq mmol⁻¹); ³H₂O, sp. act. 0.05 mCi ml⁻¹ (1.85 MBq ml⁻¹).

After incubation, 400 μ l replicate samples were taken and filtered through 0.45 μ m nitrocellulose membrane filters (Gelman Sciences). The samples were dried by suction for 2 min (but not washed) and then placed in vials containing 5 ml scintillation cocktail (Opti-fluor, Packard Instruments). In

addition, 50 μ l replicate samples were taken directly from the cell suspension and placed into vials containing 5 ml scintillation cocktail and a nitrocellulose filter disk. The vials were allowed to sit overnight at room temperature to ensure elution of the radiolabelled cells and even distribution of radioactivity from the filters. The radioactivity was then measured for 10 min using a double label program in a scintillation counter. The cell volumes were calculated using the formula described by Rottenberg (1979).

Measurement of Δ pH and $\Delta\psi$. Δ pH (pH gradient) was determined from the equilibrium distribution of [7-¹⁴C]-benzoic acid using flow dialysis (Ramos *et al.*, 1979; Friedberg & Kaback, 1980). The equilibrium distribution of benzoic acid was determined by substituting the calculated pH values into the Henderson–Hasselbalch equation, using 50 μ M benzoic acid (final concn), $pK_a = 4.19$ and an internal volume of 1.71 μ l (mg dry wt cells)⁻¹. The flow dialysis cell (model H40270, Bel-Art Products) was set at a flow rate of 4.4 ml min⁻¹ and 1 ml fractions were collected for measurement of radioactivity by liquid scintillation spectrometry. The upper and lower chambers of the flow dialysis cell were separated by a dialysis membrane with a molecular mass cutoff of 6000 Da (product H40299, Bel-Art Products). The membrane was prepared by autoclaving for 15 min in 5 mM EDTA (sodium salt, pH 7), rinsed three times in deionized water and stored in water at 4 °C until needed. Cells were grown to OD_{650} 0.4–0.5, and harvested at 4 °C. Sphaeroplasts were prepared (Godchaux & Leadbetter, 1988) and centrifuged at 2500 g for 5 min. The supernatant was removed and KCl (1 M) was added to the cell pellet (final concn 100 μ M) to enhance cell stability. To determine the Δ pH, the sphaeroplast suspension was added to the upper chamber of the flow dialysis cell (final concn 2 mg dry wt cells ml⁻¹) containing 0.1 M potassium phosphate buffer pH 6.0 (total volume of upper chamber, 800 μ l). The flow was started and radioactive benzoic acid [final concn 50 μ M, sp. act. 13.6 mCi mmol⁻¹ (0.5 GBq mmol⁻¹)] was added to the upper chamber. Both upper and lower chambers were mixed thoroughly with stirbars. After 25 fractions had been collected, 10 μ M valinomycin was added (when used), and after fraction 35 had been collected, 10 μ M nigericin was added. Since sphaeroplasts contained ample endogenous substrate, ascorbate and PMS were not needed as an electron donor system. Agents to be tested were added to the upper chamber with the sphaeroplast suspension prior to the addition of benzoate. The same buffer as in the upper chamber was pumped through the lower chamber (via a peristaltic pump) to a fraction collector and the radioactivity in the effluent was determined.

The electrical potential across the membrane ($\Delta\psi$) was determined by measuring the equilibrium distribution of TPP⁺ using flow dialysis, as described for Δ pH, with slight modifications. The flow rate of the buffer through the flow dialysis cell was reduced to 3.6 ml min⁻¹. Ascorbate (20 mM), PMS (0.1 mM) and MgSO₄ (10 mM) were added to the upper chamber of the flow dialysis cell containing the sphaeroplast suspension (final concn 4 mg dry wt cells ml⁻¹) in 0.2 mM potassium phosphate buffer with 150 mM NaCl at pH 7.5. The flow was started and the reaction was initiated after the addition of 10 μ M TPP⁺ [sp. act. 37 Ci mmol⁻¹ (1.4 TBq mmol⁻¹)]. Equilibrium was established, and after 50 fractions had been collected, the appropriate concentration of agent to be tested was added. At fraction 80, 10% Triton X-100 was added to ensure complete disruption of the cell membrane. No correction was made for binding of TPP⁺ to the surface of the sphaeroplasts since binding was never greater than 5% of the radioactivity measured.

Calculation of ΔpH , $\Delta\psi$ and protonmotive force (Δp). The equilibrium distribution of solutes was calculated using the measured value of $1.71 \mu\text{l}$ internal volume ($\text{mg dry wt cells}^{-1}$). The internal pH was calculated as described by Ramos *et al.* (1979) and the pH gradient was expressed as the difference between the internal and external pH. This value can be converted into mV by substitution into the Nernst equation. The membrane potential ($\Delta\psi$) was also calculated by substituting the equilibrium concentrations of TPP^+ across the membrane into the Nernst equation. The protonmotive force (Δp) was determined by substituting the values of ΔpH and $\Delta\psi$ into the equation $\Delta p = \Delta\psi - 59 \Delta\text{pH}$, where $\Delta\psi$ is the membrane potential in mV, ΔpH is the proton gradient across the membrane and Δp is the protonmotive force expressed in mV. The constant, 59, in the equation equals $2.303 RT/F$ at 25°C .

RESULTS

Effects on colony spreading, movement of latex beads and raft movement

Colony spreading. When diluted cell suspensions of *C. johnsonae* were plated onto solid media containing concentrations of sodium acetate ranging from 0 to 85 mM, typical colony spreading was observed up to 50 mM. However, with 50 mM acetate, both spreading and non-spreading colonies were observed. On media containing >65 mM acetate, the colonies that appeared were non-spreading and the initial viable count on this medium was markedly reduced when compared to counts using media with <50 mM acetate. In the continuation of this study, we employed a concentration of acetate (45 mM) at which colony spreading was normal.

Since a decrease in the electrochemical gradient in the membrane by acetate (or any of the 'energy-related' agents) could conceivably diminish the ability of cells to glide (or colonies to spread), we examined the effect of several such agents on colony spreading by incorporating increasing concentrations of them into agar medium (Table 1). We found that in addition to acetate,

recognized inhibitors such as arsenate, azide and cyanide also permitted growth and colony spreading, whilst DNP, CCCP, FCCP and DCCD inhibited colony spreading at the concentrations listed (Table 1).

Movement of latex beads. Bead movement was totally inhibited on cells grown on medium containing acetate. In addition, fewer beads appeared to be bound to the surface of acetate-grown cells than noted for control cells (data not shown). Within a fraction (about one-third) of a generation time, bead movement would resume on cells that had been washed free of acetate and resuspended in broth lacking acetate. Control cells (grown in broth without acetate, then centrifuged, washed and resuspended in acetate broth) ceased to move beads within 5 min of exposure to acetate.

As shown in Table 1, bead movement was not observed on cells grown in the presence of FCCP, cyanide or arsenate; the number of beads bound to the cell surface was estimated to be reduced by approximately 40%. Not only was bead movement stimulated in cells grown with 50 μM DNP, but twice that amount did not inhibit the movement of beads on the cell surface. Bead movement continued on cells exposed to azide and CCCP even after 5 h of exposure to the inhibitor. When concentrations of DCCD and CCCP were increased to 15 μM and 10 μM , respectively, bead movement stopped on control cells (grown in broth without acetate, centrifuged, washed and then suspended in broth with acetate) within 5 min of exposure.

Since bead movement has normally been assessed using liquid-grown cells, we sought to determine if beads could move on cells that had been grown on agar surfaces. We sought also to determine the effect that longer exposure to acetate might have on bead movement. Cells were plated on agar containing acetate, incubated for 48 h, rinsed from the plates and resuspended in broth with and without acetate. Cells were monitored for up to 3 h for bead movement on their

Table 1. Inhibitors, concentrations and their effects on *Cytophaga johnsonae*

Inhibitor	Concn range tested	Selected concn	Generation time (min)	Bead movement*	Raft movement*	Spreading on agar†	Presumed inhibitory effect
None			105	+	+	S	
Acetate	0–85 mM	45 mM	127	–	+	S	Protonophore
DNP	5–400 μM	50 μM	115	+	+	NS	Protonophore
CCCP	0.1–10 μM	5 μM	180	+	–	NS	Protonophore
FCCP	1–10 μM	2.5 μM	90	–	–	NS	Protonophore
Azide	10 μM –30 mM	100 μM	120	+	+	S	Terminal oxidase
Cyanide	1–3 mM	1 mM	360	–	+	S	Terminal oxidase
Arsenate	10–40 mM	20 mM	130	–	+	S	ATPase (competes for P_i)
DCCD	5–15 μM	10 μM	126	+	+	NS	F_0 ATPase

* Determined from >20 replicates.

† S, growth and colony spreading; NS, growth but no spreading.

Table 2. Rate of raft movement in the presence of inhibitors

Time (min)	Acetate		DNP		CCCP		FCCP		Azide		Cyanide		Arsenate		DCCD	
	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+
0*	100	81	100	78	100	0	100	0	100	127	100	52	100	162	100	181
5†	124	73	124	45	126	0	140	0	130	58	175	40	114	129	140	59
10	158	79	79	46	137	0	125	0	208	76	118	53	102	142	125	39
15	159	62	111	55	106	0	145	0	163	60	110	44	136	145	145	58
30	126	37	140	63	98	0	121	0	134	42	124	60	77	155	121	87

* Cells were grown in the absence of inhibitor and observed on agar with (+) or without (–) the inhibitor.

† Cells were grown in the presence of inhibitor and observed on agar with (+) and without (–) inhibitor. Data are expressed as a percentage of raft movement of control cells grown in the absence of the inhibitor and examined on agar without inhibitor. Each value represents the mean of three or more separate experiments.

surfaces. Cells grown in the absence of acetate moved beads immediately (control). However, bead movement was not observed on cells grown with acetate then placed in broth containing acetate at the same concentration. In contrast, bead movement did resume 1 h after cells grown on agar with acetate were resuspended in broth lacking acetate.

Ability of cells to move as rafts. The ability of cells to coalesce, and form and move as groups of cells (rafts) was assessed at various time points after exposure to each of the test agents. The rate of raft movement was calculated and expressed as a percentage of the control rate (Table 2).

Acetate had little inhibitory effect on raft movement, whilst CCCP and FCCP totally prevented raft formation. These cells were not dead; when washed free of the inhibitor, they formed rafts when deposited onto agar lacking CCCP or FCCP. In contrast, arsenate increased the rate of raft movement to well above control levels. Other agents (e.g. DNP, azide, cyanide) decreased the rate of raft movement to approximately 50% of the control rate. An initial decrease in raft movement was noted at 5 min but little change occurred in the ensuing 30 min. NaCl was used in this particular set of experiments as an additional salt control (osmolyte). No significant difference in the rate of raft formation was observed in the presence of 50 mM NaCl (data not shown).

Effects on O₂ consumption

Fig. 1(a) illustrates the effect that the various compounds tested had on the rate of O₂ consumption in *C. johnsonae* at 25 °C. Most slowed the rate of O₂ consumption (CCCP, FCCP, azide, cyanide) or had little or no effect (arsenate, DCCD).

Although CCCP and FCCP presumably act to disrupt the membrane potential, as well as to uncouple oxidative phosphorylation, they decreased the O₂ consumption by only 26% and 37%, respectively, at the concentrations tested. Azide and cyanide dramatically decreased O₂ consumption by ≥85%. The apparent stimulation of

respiratory activity by acetate and DNP was not statistically significant (Kruskal–Wallis test of significance).

Since the effects of these agents on *C. johnsonae* were in some instances surprising, we assessed their effects (at the concentrations employed for *C. johnsonae*) on *E. coli* grown at 37 °C and 25 °C (Fig. 1b). Clearly the effects of the agents on *C. johnsonae* and *E. coli* were not identical for each agent. At the concentrations tested, the agents decreased O₂ utilization in *E. coli*; an exception was arsenate, which had no effect at 25 °C but produced a 13% stimulation at 37 °C. DCCD, which had no effect on *C. johnsonae*, did affect *E. coli*, leading to a decrease in O₂ consumption of 60%. While O₂ consumption in *E. coli* at 37 °C was inhibited approximately 32% by acetate and 42% by DNP, O₂ consumption was not affected in *C. johnsonae* by either compound at the concentrations employed.

Effect on cellular ATP content

Fig. 2 illustrates the effects of the various agents on the total cellular ATP in growing cultures of *C. johnsonae* when exposed for up to 30 min. The optical densities were measured at the start and finish of the experiment for the purpose of monitoring growth over the 30 min test period. The initial OD₆₅₀ was 0.17. Optical densities were greater at termination than at the onset of the experiment (thus confirming growth), with the exceptions of cultures containing DNP (OD₆₅₀ 0.22), DCCD and azide (both OD₆₅₀ 0.20), where growth was seriously affected by the action of the various inhibitors. Not surprisingly, the most potent inhibitors appeared to be cyanide and arsenate.

Fig. 2(a) demonstrates the effect of protonophores presumed to act on *C. johnsonae* by inducing a proton flux in the membrane by rendering it 'leaky' to protons. As a result, the membrane would not have been able to maintain a proton gradient; ATP can still be produced, but not as efficiently. Cultures to which CCCP, FCCP or acetate were to be added contained 2.34, 2.40 and 2.23 nmol ATP (mg dry wt cells)^{–1}, respectively. After

Fig. 1. Effect of inhibitors of electron transport and oxidative phosphorylation on O_2 consumption. Each bar represents the mean of three values and was compared to a control consisting of cells in the same medium without inhibitor. The error bars represent the standard error of the mean. (a) O_2 consumption by *C. johnsonae* grown at 25 °C; (b) O_2 consumption by *E. coli* grown at 37 °C (open bars) and 25 °C (filled bars).

Fig. 2. Mean total ATP concentration in *C. johnsonae* cells after addition of (a) various protonophores and (b) various ATPase and terminal oxidase inhibitors. Each value represents three or more individual assessments. The error bars represent the standard error of the means. The optical density of the culture was determined before the onset of the assay and was adjusted to OD₆₅₀ 0.17 (zero time). Final optical densities were measured at the termination of the assay (30 min) and are indicated at the right of the graph. (a) ○, Control; □, FCCP; ●, acetate; ■, DNP; ▲, CCCP. (b) ○, Control; □, DCCD; ●, arsenate; ■, cyanide; ▲, azide.

30 min exposure to the test agent, the ATP levels were dramatically reduced [0.91, 1.10 and 0.71 nmol ATP mg (dry wt cells)⁻¹, respectively]. Although its mode of action is thought to be the same as the above agents, DNP had little effect on total cellular ATP. Initial values of 2.28 nmol ATP (mg dry wt cells)⁻¹ and final values of 2.23 nmol ATP (mg dry wt cells)⁻¹ were seen with DNP, as compared to control values of 2.34 and 3.40 nmol ATP (mg dry wt cells)⁻¹, respectively.

Fig. 2(b) displays the effect of inhibitors of terminal oxidase (azide, cyanide) and of ATPase (DCCD, arsenate, azide) on ATP production in cells. Most pro-

nounced is the effect of cyanide on ATP production. Even though the ATP pool at the onset was 2.46 nmol (mg dry wt cells)⁻¹, the effect of cyanide was immediate and nearly complete, resulting in only 0.08 nmol ATP (mg dry wt cells)⁻¹ after a 30 min exposure. Interestingly, azide, also an inhibitor of terminal oxidases as well as an inhibitor of the F₁ subunit of ATPase, did not have as profound an effect as cyanide. The initial ATP value with azide was 2.51 nmol (mg dry wt cells)⁻¹, but fell only to 1.31 nmol (mg dry wt cells)⁻¹, slightly less than one-third that of the control, within the 30 min

Table 3. Summary of the effect of inhibitors on ΔpH , $\Delta\psi$ and Δp of *Cytophaga johnsonae*

Inhibitor	ΔpH^*		$\Delta\psi^\dagger$ (mV)	$\Delta p^\ddagger$ (mV)
	at pH 6.0	at pH 7.5		
None	1.4 ± 0.14	0	-130 ± 18	-130
Acetate	0	0	-101 ± 15	-101
DNP	0	0	-80 ± 15	-80
CCCP	0	0	-104 ± 5	-104
Azide	0.78 ± 0.14	0.72	-119	-161
Cyanide	0	0	-110 ± 4	-110
Arsenate	0.96 ± 0.20	0.54	-94 ± 9	-126
DCCD	0.75 ± 0.14	0.75	-83 ± 7	-127

* Determined in 0.1 M phosphate buffer.

† Determined in 0.2 mM phosphate buffer at pH 7.5.

‡ Values given are for cells growing at pH 7.5. FCCP was used to dissipate the membrane potential with the control cells.

exposure. Azide treatment decreased the ATP content from 2.5 to 1.31 nmol (mg dry wt cells)⁻¹ within 30 min exposure. Arsenate, which is a competitor of the phosphorylation of ADP to ATP, reduced ATP levels from 2.48 to 0.39 nmol ATP (mg dry wt cells)⁻¹. DCCD, an inhibitor of the F_0 subunit of ATPase, initially decreased ATP production at a steady rate, but after 15 min ATP levels began to rise gradually. This unexpected rise in ATP levels was reproducible. In the control, the optical density as well as the concentration of ATP was increased.

Effect of inhibitors on ΔpH and $\Delta\psi$

Calculations based upon the equilibrium distribution of benzoic acid using flow dialysis before and after the addition of valinomycin indicated an internal pH of 7.45 and 7.63, respectively (plot not shown). The ΔpH without valinomycin was 1.45 pH units (7.45 inside, 6.0 outside), and the ΔpH with valinomycin was 1.63 pH units (7.63 inside, 6.0 outside). When substituted into the Nernst equation, these values before and after the addition of valinomycin corresponded to 86 mV and 97 mV, respectively.

As expected, the proton gradient was collapsed by the protonophores DNP, CCCP and FCCP, resulting in a pH_{in} equal to the pH_{out} (Table 3). Acetate (45 mM) also acted as a protonophore by collapsing the proton gradient. However, at concentrations of acetate ≤ 10 mM, a small ΔpH was observed (data not shown). Cyanide, although not considered a protonophore, also collapsed the proton gradient. Cells exposed to azide, arsenate and DCCD maintained a slight proton gradient, with pH_{in} of 6.78, 6.96 and 6.75, respectively.

Using flow dialysis procedures similar to those described for ΔpH , $\Delta\psi$ was determined by measuring the equilibrium distribution of the lipophilic cation, TPP⁺.

Addition of some test agents resulted in a rapid release of TPP⁺ from the cell interior, presumably as a result of the loss of the electrical potential in the membrane. Control cells maintained a $\Delta\psi$ of -130 mV, as shown in Table 3, and other agents lowered the $\Delta\psi$ only modestly.

DISCUSSION

The results of the present study supply new evidence that the ability of cells of *C. johnsonae* to move beads over their surface is not necessarily correlated with the mechanics of gliding and colony spreading. Although acetate is unable to serve as a sole source of carbon and energy for growth of this bacterium, when supplied at nutrient-level concentrations this compound stopped bead movement on bacterial surfaces without completely inhibiting either raft formation or movement or typical colony spreading on the agar-solidified medium. The basis for this differential inhibition of bead movement and cell migration, two events which many assume to be causally related, has been a focus of this report. We compared the effects of acetate, a weak acid and recognized protonophore, with the effects of several chemicals considered to interfere with energy production in cells (i.e. electron transport or protonmotive force).

The ability of the cytophagas to move latex spheres over their cell surface is not an ability widespread among prokaryotes. Accordingly, the observations that certain mutant cytophagas unable to move beads were also unable to glide (Pate & Chang, 1979) led to the suggestions (see also Lapidus & Berg, 1982) that bead movement was a reflection of the operation of the motility machinery responsible for cell migration. This attractive relationship has, however, been considered tenuous (Ridgway & Lewin, 1988; Gorski *et al.*, 1991) as a result of additional studies. We conclude, on the basis of separate studies, that the ability of this

bacterium to move beads over its surface is not a necessary correlate of cell migration with the resultant formation and movement of rafts of cells and the formation of thin, uneven-edged spreading colonies characteristic of this gliding bacterium.

The results presented here support the notion that the existence of a membrane potential, rather than ATP *per se*, supplies the energy for prokaryotic gliding motility (e.g. Pate & Chang, 1979; Ridgway, 1977; Chailakhyan *et al.*, 1982; Glagoleva *et al.*, 1980). They also suggest that ATP content has a more significant effect on bead movement than does the protonmotive force. The magnitude of differences in protonmotive force between control cells and those treated with test agents is minimal and accordingly seems unlikely to account for the inhibition of bead movement. In contrast, cell motility was little affected by those compounds that both stopped bead movement and brought about a pronounced decrease in cell ATP content.

It is important to note that assumptions about the effects of various classical inhibitory agents on *C. johnsonae* may be imprecise, largely because of the concentrations employed (those that permitted growth of the bacterium, rather than the usually higher concentrations employed in studies of other organisms in which growth was notably altered), and because assessment of effects on bead movement are short-term, while effects on raft movement and colony spread are measured over considerably longer time periods. Another necessary caveat is based on the profound differences between the chemical composition of the outer membrane of this bacterium and that of many other Gram-negative cells (Godchaux & Leadbetter, 1988; Pitta *et al.*, 1989), particularly in lipid composition and membrane protein profiles. One indication of the impact of these differences is reflected in the unusual inhibition of growth of gliding bacteria by actinomycin D, a sensitivity unusual among Gram-negative bacteria (Dworkin, 1969). Clearly, the actions of agents tested in this study may not be those reported for other well-studied organisms. However, any uncertainty of the actual mode of action(s) does not diminish the significance of the differential effects noted on bead movement and cell migration.

Until a definitive understanding of the mechanism(s) and machinery involved in gliding motility becomes available, any significance of the small differences noted in protonmotive force between cells grown in the presence or absence of acetate or the 'metabolic' inhibitors remains a matter of speculative interpretation. This is also the case for any relationships between bead movement and cell motility; although these do not seem to be equivalent processes, it is possible that they may share some common machinery, as has also been suggested by Gorski *et al.* (1991).

ACKNOWLEDGEMENTS

This research was supported by grant MCB-9220647 from the National Science Foundation. We also thank Robert Veith of the Biotechnology Center at the University for his time and

technical assistance in the determination of ATP, and Professors Eva Kashket and Judith Armitage for helpful comments.

REFERENCES

- Abbanat, D. R., Leadbetter, E. R., Godchaux, W., III & Escher, A. (1986). Sulfonolipids are molecular determinants of gliding motility. *Nature* **324**, 367–369.
- Chailakhyan, L. M., Glagolev, A. N., Glagolev, T. N., Murvanidze, G. V., Potapova, T. V. & Skulachev, V. P. (1982). Intercellular power transmission along trichomes of cyanobacteria. *Biochim Biophys Acta* **679**, 60–67.
- Collins, S. H. & Hamilton, W. A. (1976). Magnitude of the protonmotive force in respiring *Staphylococcus aureus* and *Escherichia coli*. *J Bacteriol* **126**, 1224–1231.
- Duxbury, T., Humphrey, B. A. & Marshall, K. C. (1980). Continuous observations of bacterial gliding motility in a dialysis microchamber: the effects of inhibitors. *Arch Microbiol* **124**, 169–175.
- Dworkin, M. (1969). Sensitivity of gliding bacteria to actinomycin D. *J Bacteriol* **98**, 851–852.
- Friedberg, I. & Kaback, H. R. (1980). Electrochemical proton gradient in *Micrococcus lysodeikticus* cells and membrane vesicles. *J Bacteriol* **142**, 651–658.
- Glagoleva, T. N., Glagolev, A. N., Gusev, M. V. & Nikitina, K. A. (1980). Protonmotive force supports gliding in cyanobacteria. *FEBS Lett* **117**, 49–53.
- Godchaux, W., III & Leadbetter, E. R. (1983). Unusual sulfonolipids are characteristic of the *Cytophaga-Flexibacter* group of gliding bacteria. *J Bacteriol* **153**, 1238–1246.
- Godchaux, W., III & Leadbetter, E. R. (1988). Sulfonolipids are localized in the outer membrane of the gliding bacterium *Cytophaga johnsonae*. *Arch Microbiol* **150**, 42–47.
- Godchaux, W., III, Gorski, L. & Leadbetter, E. R. (1990). Outer membrane polysaccharide deficiency in two nongliding mutants of *Cytophaga johnsonae*. *J Bacteriol* **172**, 1250–1255.
- Godchaux, W., III, Lynes, M. A. & Leadbetter, E. R. (1991). Defects in gliding motility in mutants of *Cytophaga johnsonae* lacking a high-molecular-weight cell surface polysaccharide. *J Bacteriol* **173**, 7607–7614.
- Gorski, L., Leadbetter, E. R. & Godchaux, W., III (1991). Temporal sequence of trait recovery during phenotypic curing of a *Cytophaga johnsonae* motility mutant. *J Bacteriol* **173**, 7534–7539.
- Hamilton, R. D. & Holm-Hansen, O. (1967). Adenosine triphosphate content of marine bacteria. *Limnol Oceanogr* **12**, 319–324.
- Lapidus, I. R. & Berg, H. C. (1982). Gliding motility of *Cytophaga* sp. strain U67. *J Bacteriol* **151**, 384–398.
- Nichols, P., Stulp, B. K., Jones, J. G. & White, D. C. (1986). Comparison of fatty acid content and DNA homology of the filamentous gliding bacteria *Vitreoscilla*, *Flexibacter*, *Filibacter*. *Arch Microbiol* **146**, 1–6.
- Pate, J. L. & Chang, L.-Y. E. (1979). Evidence that gliding motility in procaryotic cells is driven by rotary assemblies in the cell envelopes. *Curr Microbiol* **2**, 59–64.
- Pate, J. L. & Ordal, E. J. (1967). The fine structure of *Chondrococcus columnaris*. I. Structure and formation of mesosomes. *J Cell Biol* **35**, 1–13.

of ornithine amino lipid content in a sulfonolipid-deficient mutant of *Cytophaga johnsonae*. *J Bacteriol* **171**, 952–957.

Ramos, S., Schuldiner, S. & Kaback, H. R. (1979). Use of flow dialysis for determination of ΔpH and active transport. *Methods Enzymol* **55**, 680–688.

Ridgway, H. F. (1977). Source of energy for gliding motility in *Flexibacter polymorphus*: effects of metabolic and respiratory inhibitors on gliding movement. *J Bacteriol* **131**, 544–556.

Ridgway, H. F. & Lewin, R. A. (1988). Characterization of gliding motility in *Flexibacter polymorphus*. *Cell Motil Cytoskeleton* **11**, 46–63.

Rottenberg, H. (1979). The measurement of membrane potential and ΔpH in cells, organelles, and vesicles. *Methods Enzymol* **55**, 547–569.

Stock, J. B., Rauch, B. & Roseman, S. (1977). Periplasmic space in

Salmonella typhimurium and *Escherichia coli*. *J Biol Chem* **252**, 7850–7862.

Walker, R. W. (1968). *Cis*-11-hexadecenoic acid from *Cytophaga hutchinsonii* lipids. *Lipids* **4**, 15–18.

Ware, J. C. & Dworkin, M. (1973). Fatty acids of *Myxococcus xanthus*. *J Bacteriol* **115**, 253–261.

Weibull, C. (1960). Movement. In *The Bacteria*, vol. I, pp. 153–205. Edited by I. C. Gunsalus & R. Y. Stanier. New York: Academic Press.

Wolkin, R. H. & Pate, J. L. (1986). Phage adsorption and cell adherence are motility-dependent characteristics of the gliding bacterium *Cytophaga johnsonae*. *J Gen Microbiol* **132**, 355–367.

Received 10 June 1997; accepted 30 June 1997.