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High speed marine bacteria use sodium-ion and proton driven motors

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ABSTRACT: The ocean's strong ionic environment may be important for motility in marine bacteria. This is because flagellar motors are powered by dissipation of ion gradients across their cell membranes. We tested how much the 2 known motor systems contributed to the high speed motility (>100 μm s^{-1}) found in marine bacterial communities and isolates. Monensin, carbonylcyanide-m-chlorophenylhydrozone (CCCP) and amiloride were used on *Escherichia coli*, *Shewanella putrefaciens*, *Alteromonas haloplanktis*, a marine isolate (BBAT1) and marine bacterial communities to uncouple sodium-ion and proton gradients from motility. *E. coli* motility was stopped by 10 μM CCCP. Use of any of the 3 uncouplers alone slowed, but did not stop, *S. putrefaciens*, *A. haloplanktis* and a community of marine bacteria. A combination of 20 μM CCCP and 20 μM monensin stopped *S. putrefaciens* and *A. haloplanktis*. The same concentration combination reduced marine community speeds by half, but stopped few cells. Above uncoupler concentrations of 30 μM speed remained unchanged at about 20 μm s^{-1} for marine bacterial communities. Sodium-ion motors were responsible for about 60% of marine bacterial speed. From the results it was concluded that most high speed marine bacterial community members used sodium and proton motors simultaneously.

KEY WORDS: Marine bacterial motors · Motility · Proton · Sodium-ion · Uncouplers

INTRODUCTION

Chemotaxis is found in many microbial assemblages and is generally acknowledged as an important means by which bacteria encounter nutrient patches (Blackburn et al. 1998). The ocean is increasingly seen as an environment of many small nutrient patches embedded in a dynamic, organic polymer matrix (Azam et al. 1994, Azam 1998). Modelling predicts that chemotactic capability of bacteria in the ocean can increase their growth rate by 50% (Blackburn et al. 1997), and experimental work suggests that at least 85% of water column marine bacteria are capable of motility (Mitchell et al. 1995a). Their movement pattern is modified from the standard 'run and tumble' strategy used by enteric bacteria such as *Escherichia coli* (Berg & Brown 1972) and soil bacteria such as *Rhizobium meleloti* (Götz et al. 1982). Modifications include reversals rather than tumbles and speeds in excess of 200 μm s^{-1}, up to 10 times faster than *E. coli* (Mitchell et al. 1995a). The mechanism of the reversals arises from polar grouping of flagella on the bacterial surface. In contrast, the mechanism and significance of high speed in marine bacteria are more complicated. Due to shear and their small size, speeds of the order of 100 μm s^{-1} are required for successful chemotaxis by marine bacteria (Mitchell 1991, Bowen et al. 1993). Since the power requirement increases as the square of the speed, the energetic cost is high in an environment where nutrient availability is low or intermittent (Morita 1988, Azam 1998). While high speed and cost may be necessary to remain near small nutrient sources in a turbulent ocean, the mechanism is little studied. To better understand the mechanism underlying high speed and chemotaxis in marine communities, this research focused on the motor types that drive marine bacterial flagella.

Flagellate bacteria swim by rotating helical flagella, each driven by a rotary molecular motor embedded in the cytoplasmic membrane and cell wall (Macnab 1987). The motors are powered by an electrochemical gradient of either protons or sodium ions. Motile bacteria can possess either or both motor types. *Escherichia*

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coli possess only proton-driven motors, alkalophilic *Bacillus* species use only sodium motors, while the marine species *Vibrio alginolyticus*, uses proton and sodium-ion driven motors (Macnab 1987, Imae & Atsumi 1989, Atsumi et al. 1996). The difference in the motors extends beyond the ions that power them. *E. coli* and other proton-driven bacteria have motors that rotate at a few hundred revolutions per second (rps) (Lowe et al. 1987, Berry et al. 1995), whereas the sodium-ion motors of *V. alginolyticus* rotate between 1000 and 1700 rps, with accompanying speeds of up to 150 μm s^{-1} (Magariyama et al. 1994). The dual motor system is believed to reflect distinct physical environments in which different bacteria live, where proton motors are used in high viscosity environments (ca 10 cP), while sodium-ion motors are used in low viscosity environments (ca 1 cP) (Atsumi et al. 1996).

The manner and extent to which double motors are used in the marine microbial community has not been evaluated. Similarly, it is unknown what fraction of the total speed for a bacterium is driven by sodium-ion motors and whether dual motor systems occur beyond the genus *Vibrio*. It is possible that a large fractional speed contribution by a sodium ion dependent motor could limit high bacterial speeds to the marine, or other high salt, environments. The objectives of the present research were to investigate the fractional speed contribution made by proton and sodium-ion motors in marine bacteria. To achieve this objective, and get the most realistic result possible, samples of natural communities were included in this study. To build a foundation for future experimental research, the same experiments were carried out on recent, but stable, isolates.

MATERIALS AND METHODS

Sampling and isolation. Samples were collected from the water column, approximately 50 m from shore, at Brighton Beach, South Australia, and kept in 500 ml, sterile, glass bottles at 21 to 23°C for 24 h. This isolated bacteria from nutrient sources, such as particles resuspended from the benthos, and was done to increase the fraction of motile cells (Mitchell et al. 1995a). To increase cell number and stimulate motility, 10% tryptic soy broth (TSB) in sterile seawater was added to produce a final concentration of 0.1% TSB. Strains of *Shewanella putrefaciens*, *Alteromonas haloplanktis* and an unidentified isolate, BBAT1, all from Brighton Beach (Mitchell et al. 1995a,b, 1996), as well as *Escherichia coli* ATCC 29922 were used for comparison with the enriched seawater community. *E. coli* was grown in 0.1% TSB broth cultures overnight while the marine isolates were grown in 0.1% TSB, autoclaved, filter sterilised seawater from Brighton Beach. The pHs for *E. coli*, the isolates and the bacterial community were 7.4 (unadjusted), 8.2 and 8.3 (adjusted with 1 M NaOH), respectively. To assess the influence of NaCl on isolate speeds, *A. haloplanktis* and *S. putrefaciens* were grown in 1, 2, 3 and 4% artificial seawater. Motility parameters were determined as described below.

Motility measurements and ion uncoupler useage. Cell speed was measured by bright-field microscopy using frame-by-frame analysis of video images (Barbara & Mitchell 1996). High speed and cluster formation were induced by the introduction of millimeter-size air bubbles underneath a coverslip containing bacteria. The percent of motile population or community was measured directly from video images according to Mitchell et al. (1995a).

The addition of ion gradient uncouplers and inhibitors, specifically the sodium-ion uncoupler monensin (10 to 60 μM) and the protonophore carbonylcyanide-m-chlorophenylhydrozone (CCCP, 3 to 30 μM) were used to determine the type of motor being used (Atsumi et al. 1992b). The sodium-ion uncoupler monensin was used in place of the more traditional amiloride for the natural communities and the 2 identified isolates because the former is less toxic to other cell functions and works at a thousandth the concentration of amiloride (Dibrov et al. 1986, Atsumi et al. 1992a). To provide a link and comparison with the amiloride literature, which established sodium-ion uncoupling (Atsumi et al. 1992a), an unidentified isolate, BBAT1, tolerant to amiloride was also included in the experiments.

Alteromonas haloplanktis and *Shewanella putrefaciens* were either grown in broth as described above with the either $MgCl_2$ or KCl substituted for NaCl at concentrations of 3.5%, or grown in standard broth, pelleted at 5000 × *g* for 5 min and then resuspended in $MgCl_2$ or KCl. In the latter case, cell speeds were recorded within 10 min of resuspension.

Transmission electron microscopy. Methods were modified from McCarter et al. (1988). Slot grids coated with 0.5% butvar were inverted for 5 min on drops of broth cultures previously fixed with 10% paraformaldehyde and 12.5% glutaraldehyde in 0.15 M sodium cacodylate buffer at pH 7.4. Grids were washed 4 times, stained for 5 s with 0.5% phosphotungstic acid, dried and examined with a Phillips 100 microscope at 80 kV.

RESULTS AND DISCUSSION

Speed alteration in an enteric standard

Escherichia coli showed no clustering behavior and moved at speeds ranging from 2 to 40 μm s^{-1} (Fig. 1).

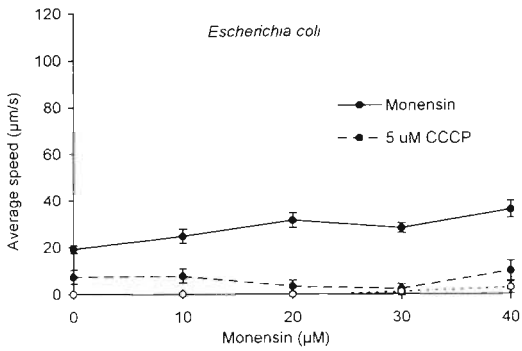

Fig. 1 Speed of *Escherichia coli* as a function of monensin concentration. Broken lines indicate the presence of both monensin and CCCP. Error bars are 95% confidence intervals. The resolution of the method is 2 µm s^{-1}

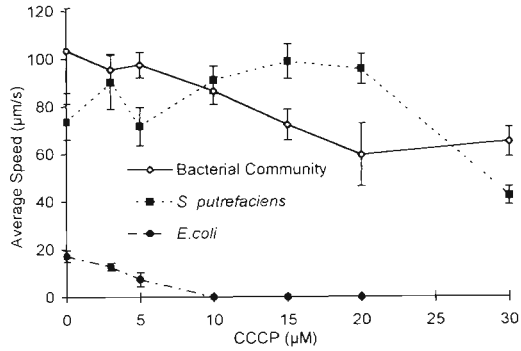

Fig. 2. Influence of CCCP on the speed of *Escherichia coli*, *Shewanella putrefaciens* and marine bacterial communities. The increasing error bars up to 20 µM in the bacterial community are believed to reflect the diversity of response to CCCP in the community. Error bars are 95% confidence intervals

The speed of *E. coli* doubled over a 40 µM change in monensin alone to approximately the maximum reported in the literature (Macnab 1987) and was reduced to zero in 10 µM CCCP, consistent with its known use of proton-driven motors (Macnab 1987). These results suggest that counter-ion transport from the 3 mM NaCl in TSB, rather than the ability of the motors to pump out sodium-ions, limited speed. The conflicting action of monensin and CCCP, at approximately equimolar concentrations, caused the decrease in speed (Fig. 1) to be less than when just CCCP was present (Fig. 2).

Speed alteration with CCCP for marine isolates and communities

The isolates, *Shewanella putrefaciens* and *Alteromonas haloplanktis*, formed 50 to 100 µm wide clusters around small air bubbles, utilising a run and reverse pattern of movement seen in the bacterial communities of this study and in previous work (Mitchell et al. 1996). Unlike *Escherichia coli*, both isolates maintained motility at CCCP concentrations above 10 µM (Fig. 2). Their sensitivities to CCCP were similar in that both showed small speed increases between 0 and 3 µM, probably due to increased counter-ion transport of sodium-ions, similar to what was observed with *E. coli* and monensin (Figs. 2 & 3). However, above 3 µM CCCP, *S. putrefaciens* maintained or increased speed up to 20 µM (Fig. 2), while *A. haloplanktis* showed an approximately linear decline in speed from 3 µM, slowing by a factor of 4 across a 5-fold concentration increase (3 to 15 µM CCCP, Fig. 3). The results indicate that a proton motor inhibitor was not sufficient to stop *S. putrefaciens* and *A. haloplanktis*, even though it slowed them down. The reason for the broad peak of speed increase in *S. putrefaciens* is unclear, but seems to indicate higher resistance to CCCP than *A. haloplanktis* possesses. The single point peak for *S. putrefaciens* at 3 µM was significantly different from 0 or 5 µM ($p = 0.005$ and $p = 0.002$, 2-tailed *t*-tests assuming unequal variance). The rise from 0 to 3 µM may be the result of a counter-ion transport phenomenon, similar to that of Fig. 1. The taxonomic level at which variation in resistance and counterion transport occurred was not ascertained, but recent work (Ziemke et al. 1997) indicates that at least *S. putrefaciens* shows considerable intraspecies genetic variability that precludes attribution of the difference to simple interspecies differences.

Overall, the results of isolates with CCCP indicated that proton driven motors were only partly responsible for their motility. This was further supported by the response of the bacterial community to CCCP. There was no observerd counter-ion speed increase in the bacterial community; rather, CCCP reduced speed by 40% from approximately 100 to 60 µm s^{-1} between 0 and 20 µM (Fig. 2), leveling out thereafter.

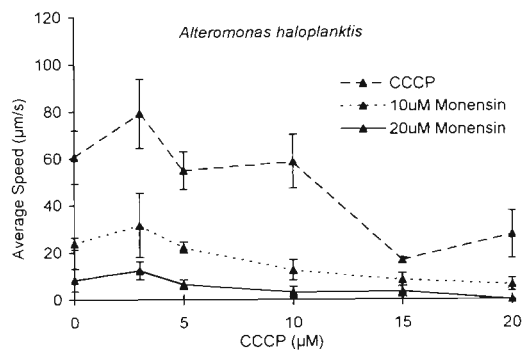

Fig. 3. Response of *Alteromonas haloplanktis* to CCCP alone and CCCP amended with 10 or 20 µM monensin. Error bars are 95% confidence intervals. Where error bars are not visible, they are smaller than the symbol

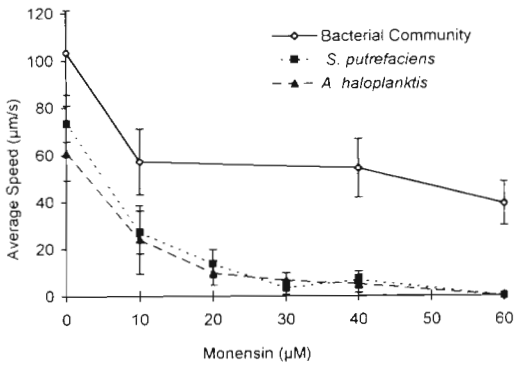

Fig. 4. Influence of monensin on the 2 marine isolates and the bacterial community. The zero micromolar monensin points serve as references for this figure and Fig. 5. Error bars are 95% confidence intervals

Speed alteration with monensin and amiloride for marine isolates and communities

The hypothesis that sodium ions were responsible for much of the isolate and community motility was tested and confirmed using monensin and amiloride. For *Alteromonas haloplanktis*, the presence of 10 and 20 µM monensin significantly reduced speed at all CCCP concentrations, compared to CCCP alone (Fig. 3). Monensin alone stopped motility in both isolates at 60 µM and reduced speed in the bacterial community by 60% (Fig. 4). A combination of 20 µM CCCP and 20 µM monensin stopped both isolates and reduced the bacterial community speed by 65% (Fig. 5). Similarly, the BBAT1 isolate in 2.5 mM amiloride showed a speed reduction of approximately 65% (data not plotted, treatment speed/initial speed = (28µm s^{-1})/(81 µm s^{-1}), 95% confidence intervals for both values = 9 µm s^{-1}).

Influence of speed alteration on motile fraction

The results presented here indicate that sodium-ion motors are responsible for at least 60% of marine bacterial speed in 1 species of *Shewanella*, 1 species of *Alteromonas*, and an unidentified isolate. The speed reduction caused by the uncouplers was probably achieved through both direct effects, on the motors, and indirect effects, by acting as a metabolic poison. *Vibrio alginolyticus*, for example, grew at pH 7, but failed to grow at pH 8.5 when amiloride was present (Atsumi et al. 1992a). From this work it appears that the balance between poisoning and specific motor inhibition is species and condition dependent. Ultimately, completely separating the 2 effects is probably impossible since a transmembrane ion gradient powers cell metabolism and 1 of the 2 motor types. The results, then, first show a species' reliance on sodium ion gradients versus proton gradients. To go beyond this, and gain at least a qualitative understanding of the balance between general poisoning and specific motor inhibition, speed against motile fraction was plotted (Fig. 6). The reasoning is that if a compound tends towards being a motor-specific inhibitor, the mean population speed will be reduced while the motile population fraction remains constant and if a compound tends towards being a general poison the fraction of motile cells in the population will be reduced as cells are killed. Visually comparing the slopes of Fig. 6a (CCCP) with those of Fig. 6b (monensin and amiloride), shows that the former appears as a specific inhibitor and the sodium-ion uncouplers appear more poisonous. This is not conclusive and may be further support that these marine bacteria are just more dependent on sodium-ion gradients than proton gradients. This may also explain why there was no difference in the slope of amiloride and monensin additions for the bacterial communities in Fig. 6b. The increase in motile fraction of *Escherichia coli* cells was likely an artefact of counter-ion transport.

Accounting for the maintenance of speed in the presence of uncouplers

Despite the speed reductions, marine bacterial communities maintained speeds of at least 30 µm s^{-1} at uncoupler concentrations that rendered the isolates immotile (Figs. 4 & 5). The maintenance of speed in the presence of uncouplers could have been caused by a low uncoupler molecule to cell ratio or by resistance to the uncouplers (Krulwich et al. 1990). An uncoupler to cell ratio was calculated by counting the number of cells per unit volume, averaging across the

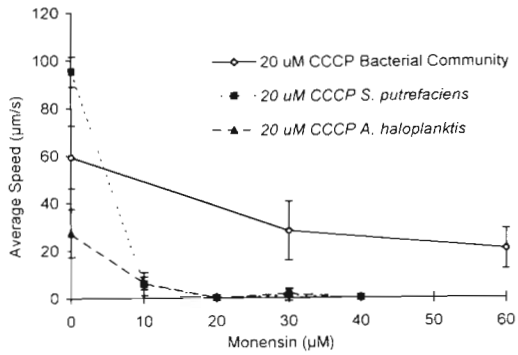

Fig. 5. Combined influence of monensin and CCCP on the speed of the isolates and bacterial community. The zero micromolar monensin points contained 20 µM CCCP and as such bacterial community speeds were similar to the 20 µM CCCP speeds, but lower than in Fig. 4, where only monensin was used. Error bars are 95% confidence intervals

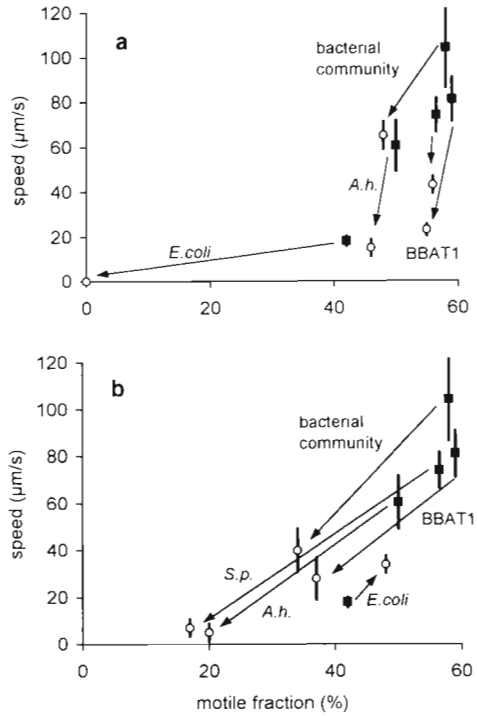

Fig. 6. Changes in mean population or community speed as a function of motile fraction. The arrows are intended to show shifts in behaviour. The interdependence of speed and motile fraction force low and zero values on either axis to the origin. Arrows indicate how both parameters changed after the addition of (a) CCCP or (b) Monensin. The response of bacterial communities to amiloride was statistically indistinguishable from the response to monensin and is not shown here for the sake of clarity. BBAT1 was tested with amiloride here for comparison with the bacterial community. Abbreviations are: *S.p.*, *Shewanella putrefaciens*; *A.h.*, *Alteromonas haloplanktis*; and BBAT1, an amiloride tolerant isolate. The *S.p.* label was left off of the small downward arrow in (a) for clarity. Error bars are 95% confidence intervals. The *x*-axis 95% confidence intervals for motile fraction were less than 15% and left off for clarity

coverslip and converting uncoupler concentration to number of molecules. There were between 10^5 and 4×10^6 uncoupler molecules per cell across all experiments. Even if most of the cell surface receptors were ion pumps, the uncouplers would still be in excess by a factor of 100 to 1000. From this we conclude that speed maintenance by the marine community took place in the presence of sufficient uncoupler to stop the cells, provided these bacteria are physiologically similar to *Escherichia coli*, *Alteromonas haloplanktis* or *Shewanella putrefaciens*. Resistance to the uncouplers seems the likely explanation for the maintenance of speed. Resistance mechanisms may include inefficient uncoupler uptake due to unusual membrane lipids (Jung et al. 1993), uncoupler binding proteins (Krulwich et al. 1990) or the presence of motors driven by ions other than sodium ions or protons. Although this latter explanation is unlikely, our data does not permit us to rule it out. Identifying the mechanism of resistance is a worthwhile goal as it is likely to shed light on how marine bacteria achieve comparatively high speeds and more broadly shed light on their physiological linkages with the marine environment.

Influence of pH, salinity and ion substitution on speed

For our isolates, pH between 7.2 and 9.2 had a pronounced impact on speed and stimulated our original interest in the balance between proton and sodium motors (Dillon 1994). The ocean is well buffered with a pH in suface waters between 8.1 and 8.3 (Millero & Sohn 1992). As the ultimate goal is to understand the function of marine bacterial communities, pH in this study was restricted to 8.2 or 8.3, except for *Escherichia coli*. Salinity is much more variable than pH in surface waters and produced ambiguous results in Dillon (1994). Here we present the influence of NaCl on speed in Fig. 7 for these same isolates. The close correlation between speed and salinity supports the inferences from the uncoupler results that sodium ion motors are important in the motility of marine bacteria. This is further supported by the ion substitution results. The isolates were moderate obligate halophiles, showing no appreciable growth or motility when $MgCl_2$ or KCl were substituted for NaCl. Resuspended *Alteromonas haloplanktis* had speed reductions in $MgCl_2$ and KCl of 70 and 49% (95% confidence intervals = 13 and 17%). Resuspended *Shewanella putrefaciens* had speed reductions in $MgCl_2$ and KCl of 56 and 38% (95% confidence intervals = 12 and 33%).

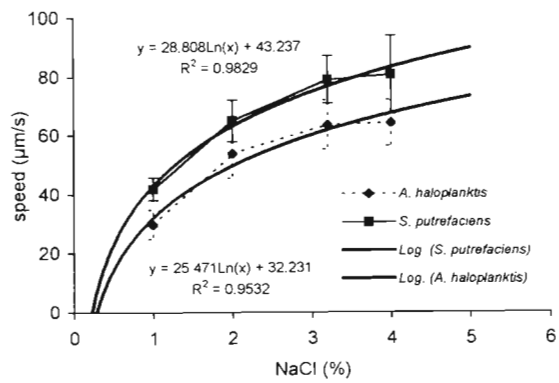

Fig. 7. Influence of sodium chloride concentration on swimming speed for 2 marine bacterial strains. Solid lines are the best fit log equations (shown with R^2 values). Error bars are 95% confidence intervals. Extrapolations are illustrative of curve shape

Interpretation

Sodium-ion motors appear to be an integral part of the high speeds observed in marine bacteria. The hypothesis supported here is that both motor types are used simultaneously. This requires, at least, that cells possess multiple flagella in broth culture. Fig. 8 confirms that *Alteromonas haloplanktis* and *Shewanella putrefaciens* possess multiple flagella in culture. To date the presence of dual motors has been identified in *Vibrio* (Magariyama et al. 1995) and here in *Shewanella*, *Alteromonas* and an isolate characterised by growth tolerance to amiloride. Furthermore, natural communities were slowed by high monensin concentrations, but unlike the isolates, showed partial resistance to its ion dissipating effects. The simultaneous use of proton and sodium-ion motors in the low viscosity environment of seawater may be necessary to reach the high speeds useful for positional control near small nutrient patches (Azam et al. 1994, Azam 1998, Blackburn et al. 1998).

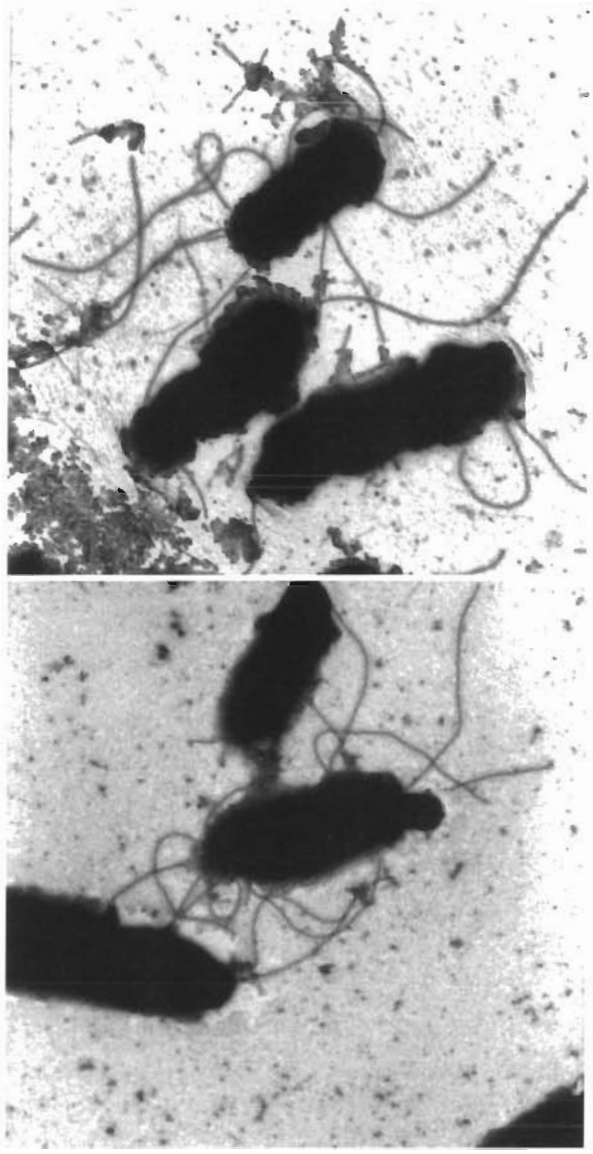

Fig. 8. TEM of (a) *Alteromonas haloplanktis* and (b) *Shewanella putrefaciens* showing multiple flagella per cell originating at polar and subpolar positions

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