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Analysis of energy sources for *Mycoplasma penetrans* gliding motility

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Abstract

Mycoplasma penetrans, a potential human pathogen found mainly in HIV-infected individuals, uses a tip structure for both adherence and gliding motility. To improve our understanding of the molecular mechanism of *M. penetrans* gliding motility, we used chemical inhibitors of energy sources associated with motility of other organisms to determine which of these is used by *M. penetrans*, and also tested whether gliding speed responded to temperature and pH. *M. penetrans* gliding motility was not eliminated in the presence of a proton motive force inhibitor, a sodium motive force inhibitor, or an agent that depletes cellular ATP. At near-neutral pH, gliding speed increased as temperature increased. The absence of a clear chemical energy source for gliding motility and a positive correlation between speed and temperature suggest that energy derived from heat provides the major source of power for the gliding motor of *M. penetrans.*

Keywords

terminal organelle; arsenate; CCCP; amiloride; microcinematography

INTRODUCTION

Cellular motility is important for a variety of processes, including obtaining nutrients, evading threats, organizing cells for developmental processes, and cell division. Both thermal and chemical energy are employed as direct sources of energy for cellular motility. For example, many eukaryotic cells are driven forward by the formation of membrane protrusions through localized polymerization of actin, powered principally by thermal energy in the form of a Brownian ratchet (Peskin *et al.*, 1993). Bacterial twitching motility is powered by ATP hydrolysis, which powers extension and retraction of type IV pili attached to a surface (Burrows, 2005). Rotation of bacterial flagella, which drive swimming and swarming movements, is powered by proton motive force (PMF) (Berg & Anderson, 1973), or rarely by sodium motive force (SMF) (McCarter, 2004). In both *Flavobacterium johnsoniae* and *Myxococcus xanthus*, gliding motility, the smooth movement of cells over a surface, is powered by PMF (Liu *et al.*, 2007; Nan *et al.*, 2010; Sun *et al.*, 2011). Since gliding motility is carried out among diverse bacterial groups and uses diverse mechanisms (McBride, 2004), no single organism can be used to model a molecular mechanism for this process.

Several mycoplasmas exhibit gliding motility, enabling these bacteria to colonize and cause infection in their hosts (Jordan *et al.*, 2007; Szczepanek *et al.*, 2012). Among these species,

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only *Mycoplasma mobile* has been studied in depth to identify its motility energy source. Arsenate, a phosphate analogue that causes depletion of cellular ATP, rapidly and potently inhibits motility of *M. mobile* (Jaffe *et al.*, 2004), and Triton X-100-permeabilized cells resume movement when ATP is added directly to the cells, demonstrating that the motor is directly dependent on ATP hydrolysis (Uenoyama *et al.*, 2005). Little is known about the energy source necessary for gliding motility in other mycoplasmas. However, it is well-established that different mycoplasma species use compositionally dissimilar tip structures for gliding motility (Relich *et al.*, 2009; Miyata, 2010; Jurkovic *et al.*, 2012), making it impossible to generalize the motility mechanisms they use.

One mycoplasma species whose gliding mechanism is unknown is *Mycoplasma penetrans*, a putative human pathogen originally isolated from the urogenital tract of HIV-positive patients (Lo *et al.*, 1991, 1992; Wang *et al.*, 1992). Its lipoproteins are mitogenic toward B and T lymphocytes (Feng *et al.*, 1994; Sasaki *et al.*, 1995) and stimulate transcription of the HIV genome *in vitro* via toll-like receptors (Shimizu *et al.*, 2004), implying a role for *M. penetrans* in the accelerated progression of AIDS. *M. penetrans* has a polar terminal organelle that leads during gliding motility and whose Triton X-100-insoluble cytoskeleton is distinct from those of most other species, including *M. mobile* (Jurkovic *et al.*, 2012). Genomic analysis reveals the absence of clear homologs of terminal organelle-associated proteins of other species (Sasaki *et al.*, 2002). The present study aims to identify potential sources of energy for gliding motility of *M. penetrans*, examining both chemical and thermal contributions to the movement of cells of this species.

MATERIALS AND METHODS

Bacterial culture conditions and passaging

M. penetrans strain HP88 was obtained through a series of passages of *M. penetrans* strain GTU-54-6A1 (Lo *et al.*, 1992) in SP-4 motility media [SP-4 broth (Tully *et al.*, 1979) supplemented with 3% gelatin]. A 100-μL aliquot of *M. penetrans* strain GTU-54-6A1 was added to 2 mL of SP-4 motility medium in a 24-well plate (TPP Techno Plastic Products AG). Upon a colour change in the medium from red to yellow, a 100-μL aliquot of the passaged *M. penetrans* was taken from the top of the well and transferred to a fresh 2 mL of SP-4 motility medium in the adjoining well. This process was repeated 75 times, generating strain HP88, which was subsequently cultured at 37°C in SP-4 broth or on SP-4 agar plates. As a control, *M. mobile* strain 163K (Kirchoff & Rosengarten, 1984) was cultured at room temperature in SP-4 broth or SP-4 motility medium.

Time-lapse microcinematography

For motility assays of *M. penetrans*, a concentrated motility stock was made by growing 50 mL of culture to mid-log phase, indicated by a colour change in the medium from red to orange. Cells were harvested by centrifugation (17,400×*g*) at 4°C for 20 min, suspended in 2 mL fresh SP-4 broth, and passed through a 0.45-μm filter before aliquoting and storage. For motility assays at various temperatures and pH, HP88 motility stocks were thawed and inoculated into SP-4 motility medium with a pH of 5.8, 6.8, 7.8, or 8.8 and incubated at 30°C, 37°C, or 40°C for 3 h before analysis. To determine the average gliding speed of *M. penetrans* HP88, excluding rest periods, cells from frozen, mid-log phase stocks were passed through a 0.45-μm filter and incubated for 3 h at 37°C in glass chamber slides (Nunc) in SP-4 motility medium, and microcinematographic analysis was performed as previously described (Hatchel *et al.*, 2006).

Energy source assays

To determine the effects of inhibitors of ATP metabolism and ion motive force on *M. penetrans* motility, cells were analyzed in buffers with or without the test reagent. *M. penetrans* motility stocks were incubated in SP-4 motility medium for 3 h at 37°C in a glass chamber slide. *M. mobile* cells from frozen mid-log phase growth were syringed 10 times before incubation in SP-4 motility media for 1 h at 25°C. For both species, the medium was then removed and each chamber was rinsed 5 times with the control or test buffer, incubated in the control or test buffer for 1 h, and analysed for motility as described above. The following buffers were used: phosphate-buffered saline supplemented with gelatin and glucose (PBS-G^2; 150 mM NaCl, 32 mM NaH_2PO_4, 136 mM Na_2HPO_4, 10 mM glucose, 3% gelatin, pH 7.2); arsenate-buffered saline supplemented with gelatin and glucose (ArBS-G^2K; 140 mM NaCl, 75 mM KCl, 10 mM glucose, 2.5 mM potassium arsenate, 4.75 mM sodium arsenate, 3% gelatin, pH 7.2); PBS-G^2 supplemented with potassium (PBS-G^2K; 140 mM NaCl, 10 mM KCl, 10 mM glucose, 50 mM sodium phosphate, pH 7.2); PBS-G^2 supplemented with CCCP [C^3PBS-G^2; 150 mM NaCl, 3.2 mM NaH_2PO_4, 13.6 mM Na_2HPO_4, 10 mM glucose, 3% gelatin, 10 μM CCCP in dimethyl sulfoxide (DMSO), pH 7.2]; and PBS-G^2 supplemented with amiloride (APBS-G^2; 150 mM NaCl, 3.2 mM NaH_2PO_4, 13.6 mM Na_2HPO_4, 10 mM glucose, 3% gelatin, 10 μM amiloride, pH 7.2). All reagents were purchased from Sigma Aldrich.

Temperature/pH study

Motility stocks were incubated in SP-4 motility medium with the desired pH (5.8, 6.8, 7.8, 8.8) and temperature (30°C, 37°C, 40°C) in glass chamber slides. For motility analysis, 18 images were captured at 1000X magnification on a Leica DM IRB inverted phase-contrast/epifluorescence microscope at approximately 0.25-s intervals. Images were merged and analysed for 20–25 motile cells as previously described (Hatchel *et al.*, 2006).

Statistical analysis

The temperature and pH data were analyzed using two-factor factorial analysis of variance (ANOVA) to examine the effects of both temperature and pH on motility speed. To determine the temperature and pH associated with maximal gliding speed, a statistical response surface model was fit to the data with an accompanying canonical analysis. The effects of energy source inhibitors on motility were analyzed by ANOVA. All statistical analyses were performed using SAS version 92 for Windows.

RESULTS

Gliding speed and HA

The advantage of using a fast-gliding strain for analysis of motility-associated phenomena is that increased gliding speed allows clearer resolution of changes in speed under different conditions. High passage *M. penetrans* strain HP88 glided in one direction with an average speed of 1201 ± 326 nm s^{-1} (n=103), twice as fast as strain GTU-54-6A1 and >20 times faster than strain HF-2 (Jurkovic *et al.*, 2012). The gliding speed of this strain, which was used for all experiments, spanned a range of 158–2115 nm s^{-1}, corresponding to 0.2–1.8 times the average gliding speed (Figure 1). For subsequent experiments, values were normalized to the gliding speed observed at 37°C and pH 7.8 in the appropriate control buffers.

Effect of arsenate on motility and growth

Arsenate enters prokaryotic and eukaryotic cells via phosphate transporters (Rosen, 2002) and inhibits many reactions involving phosphate. These reactions include substrate-level

phosphorylation events leading to ATP synthesis via the glycolysis (Warburg & Christian, 1939) and arginine dihydrolase (Knivett, 1953) pathways, the only two means of ATP synthesis available to *M. penetrans* (Lo *et al.*, 1992; Sasaki *et al.*, 2002), as mycoplasma membrane ATP synthase actually hydrolyses ATP to create a proton gradient (Linker & Wilson, 1985). To confirm toxicity of arsenate to *M. penetrans*, cells were cultured in the presence of 10 mM sodium arsenate or sodium phosphate, pH 7.2. After 2 d of incubation at 37°C, growth of *M. penetrans* was observed with added sodium phosphate, but not arsenate, (not shown), confirming that *M. penetrans* takes up arsenate and its growth is inhibited at relatively low arsenate concentrations. As mycoplasmas lack electron transport chain-associated respiration, arsenate toxicity can be attributed to ATP depletion.

Arsenate was added to *M. penetrans* cells to determine whether ATP hydrolysis by a motor-associated component directly provides energy for gliding, as proposed for *M. mobile*, upon whose gliding motility arsenate has an immediate negative impact (Jaffe *et al.*, 2004). *M. penetrans* continued to glide in the presence of 50 mM arsenate, five times the amount in which growth was prevented (see above), at incubation times ranging from 1 to 8 h. In 50 mM arsenate, the gliding speeds of both *M. mobile* [$F(1, 144)=13331$, $p<0.0003$] and *M. penetrans* [$F(1, 144)=7670$, $p<0.0003$] were significantly reduced. However, the 37% decrease for *M. penetrans* was much smaller than that of *M. mobile*, which exhibited an 89% decrease in speed (Figure 2), essentially in agreement with the observations of an absence of *M. mobile* cells moving faster than 10% of normal gliding speed after 10 min under similar conditions (Jaffe *et al.*, 2004). Although the change in speed of *M. penetrans* was statistically significant, the moderate value of the decrease and the continued movement of the cells after 8 h (not shown) suggest that direct inhibition of the motor by ATP depletion was unlikely. Increasing the arsenate concentration fivefold further, to 250 mM, had a negligible effect on *M. penetrans* motility (Figure 2). Thus, ATP hydrolysis is an unlikely energy source for gliding by *M. penetrans*.

Effect of CCCP and amiloride on motility

The presence of membrane potential has been reported in a variety of mycoplasma species (Benyoucef *et al.*, 1981; Schiefer & Schummer, 1982). To determine whether PMF supplies the energy needed for *M. penetrans* gliding motility, we observed motility in the presence of the ionophore CCCP, which collapses the proton gradient. Cells were incubated for 1 h in the presence of 10 mM CCCP in DMSO and in PBS-G^2K containing the same volume of DMSO used in the test buffer. After 1 h, gliding speed actually increased by 29% compared to the control buffer ($p<0.0001$) (Figure 2), ruling out PMF as an energy source for gliding motility of *M. penetrans.* To test SMF as a potential energy source for *M. penetrans* gliding, cells were observed in the presence of amiloride, an inhibitor of Na^+/H^+ antiporters and sodium channels which competes with Na^+ in the medium (Benos, 1982). *M. penetrans* gliding speed was not significantly affected by 1 h of incubation in amiloride ($p=0.6$) (Figure 2), ruling out SMF as an energy source.

Effects of pH and temperature on gliding motility

To determine the role of thermal energy in the motility mechanism of *M. penetrans*, we analysed its gliding speed under conditions of differing temperature. If radiant energy from ambient heat is a significant power source, then we would predict increased speed even at temperatures in excess of those normally encountered physiologically. We analysed gliding speed at temperatures ranging from 30°C to 40°C and pH levels ranging from 5.8 to 8.8 (Figure 3). Speed increased with temperature, but at acidic or alkaline pH, the trend was less distinct. Additionally, speed was greater closer to neutral pH. The interaction between temperature and pH was significant [$F(6,283)=989$, $p<0.0001$], suggesting that the effects of temperature depend on the pH. To determine the temperature and pH parameters for

maximal speed, a statistical response surface model was fitted to the data obtained from the temperature and pH assays, along with accompanying canonical analysis (Figure 4). There were highly significant linear and curvilinear effects, as well as a marginally significant interaction effect of both temperature and pH, and both were found to be significant contributors to gliding speed. The surface model revealed a rising ridge along the temperature gradient, suggesting that maximal speed occurs at a temperature higher than 40°C. Ridge analysis suggested that maximal speed was well maintained near neutral pH levels, and was found on a strongly linear trajectory in increasing temperature. At 45°C almost no cells adhered, marking 40°C as an upper limit to the experiment. These data suggest that thermal energy is limiting for gliding speed as long as the adherence and motility machinery is capable of functioning.

DISCUSSION

Energy source for *M. penetrans* gliding motility

The molecular mechanism of *M. penetrans* gliding motility is unknown and no homologues of known motility proteins in the better-characterized species, *M. pneumoniae* and *M. mobile*, are present. In an effort to identify the energy source used to power gliding, the motility behavior of *M. penetrans* was observed in the presence of chemical inhibitors previously used to characterized motility energetics in other species of mycoplasmas and bacteria. Arsenate did not have the same degree of impact on *M. penetrans* gliding as it did on *M. mobile*, with a much smaller reduction in speed. Furthermore, *M. penetrans* cells were still able to glide well after 8 h in the presence of arsenate, and at concentrations fivefold greater than those tested for *M. mobile*, both of which are conditions under which ATP is nearly completely depleted through inhibition of the reactions catalysed by glyceraldehyde 3-phosphate dehydrogenase (Warburg & Christian, 1939) and ornithine carbamoyltransferase (Knivett, 1954). As mycoplasma membrane ATP synthase actually operates in reverse to maintain a proton gradient functioning in sodium extrusion and cell volume maintenance (Linker & Wilson, 1985), and is therefore not involved in ATP synthesis, it is overwhelmingly likely that ATP is depleted under our experimental conditions, which include incubation in 25 times the concentration of arsenate that prevents growth. These data suggest that ATP hydrolysis is at best an indirect source of energy for motility in *M. penetrans*, perhaps only providing the energy necessary to replenish less stable molecular components of the motor and/or to maintain these components, such as by phosphorylation, which is essential for normal function of motility-associated proteins in *M. pneumoniae* (Schmidl *et al.*, 2010). Furthermore, neither a PMF inhibitor nor an SMF inhibitor decreased gliding speed. We therefore could not identify a convincing source of chemically derived energy for gliding.

Temperature and pH effects on gliding motility

To examine the possibility of a thermal component to the energy source for gliding, motility was observed under different temperature and pH conditions. We found that at any tested temperature, the pH optimum was between 6.8 and 7.8, although even at pH of both 5.8 and 8.8 the gliding speed was still substantially greater than the previously reported speed for strain HF-2 (Jurkovic *et al.*, 2012). At near-neutral pH, there was a clear increase in gliding speed with increasing temperature, even though normal physiological temperature was exceeded at 40°C. Therefore, near neutral pH, there is a linear relationship between temperature and motility speed. These data suggest that thermal energy is a substantive energy source for *M. penetrans* gliding motility, whereas a chemical energy source typically observed for bacterial motility was not identified. Given the role of gliding in *M. penetrans* cell division (Jurkovic *et al.*, 2012), it is conceivable that the difference in gliding speed between strains GTU-54-6A1, isolated from the urine, and HF-2, isolated from the

respiratory tract, is attributable to selection for sufficient speed at the lower pH of the urogenital tract environment.

Mechanism of *M. penetrans* gliding motility

Two models have been proposed for gliding motility in *M. mobile* and *M. pneumoniae*, the centipede and inchworm model, respectively (Miyata, 2010). In the better elucidated centipede model, adhesins reversibly bind substrate in a manner dependent upon ATP hydrolysis. There is no direct evidence in support of a particular motility model in *M. pneumoniae*, but the inchworm model has been proposed based on electron cryotomography data. In this model, flexing of the cytoskeleton within the attachment organelle causes the displacement and association of adhesins to the cell surface, moving the cell forward (Henderson & Jensen, 2006). Although it remains unclear whether either of these occurs in *M. penetrans*, our data indicate that the mechanism of motility has an important thermal component. *M. mobile* speed also correlates positively with temperature (Miyata & Uenoyama, 2002), but in that organism ATP hydrolysis is absolutely required for movement (Jaffe *et al.*, 2004; Uenoyama *et al.*, 2005), unlike in *M. penetrans*. If *M. penetrans* gliding motility is in fact driven by a Brownian ratchet mechanism that converts thermal energy into forward movement, then this is unique among prokaryotes, and suggests the existence of a yet uncharacterized cytoskeletal component capable of polarized polymerization and depolymerization. Further investigation of the structure and composition of the *M. penetrans* motor is warranted.

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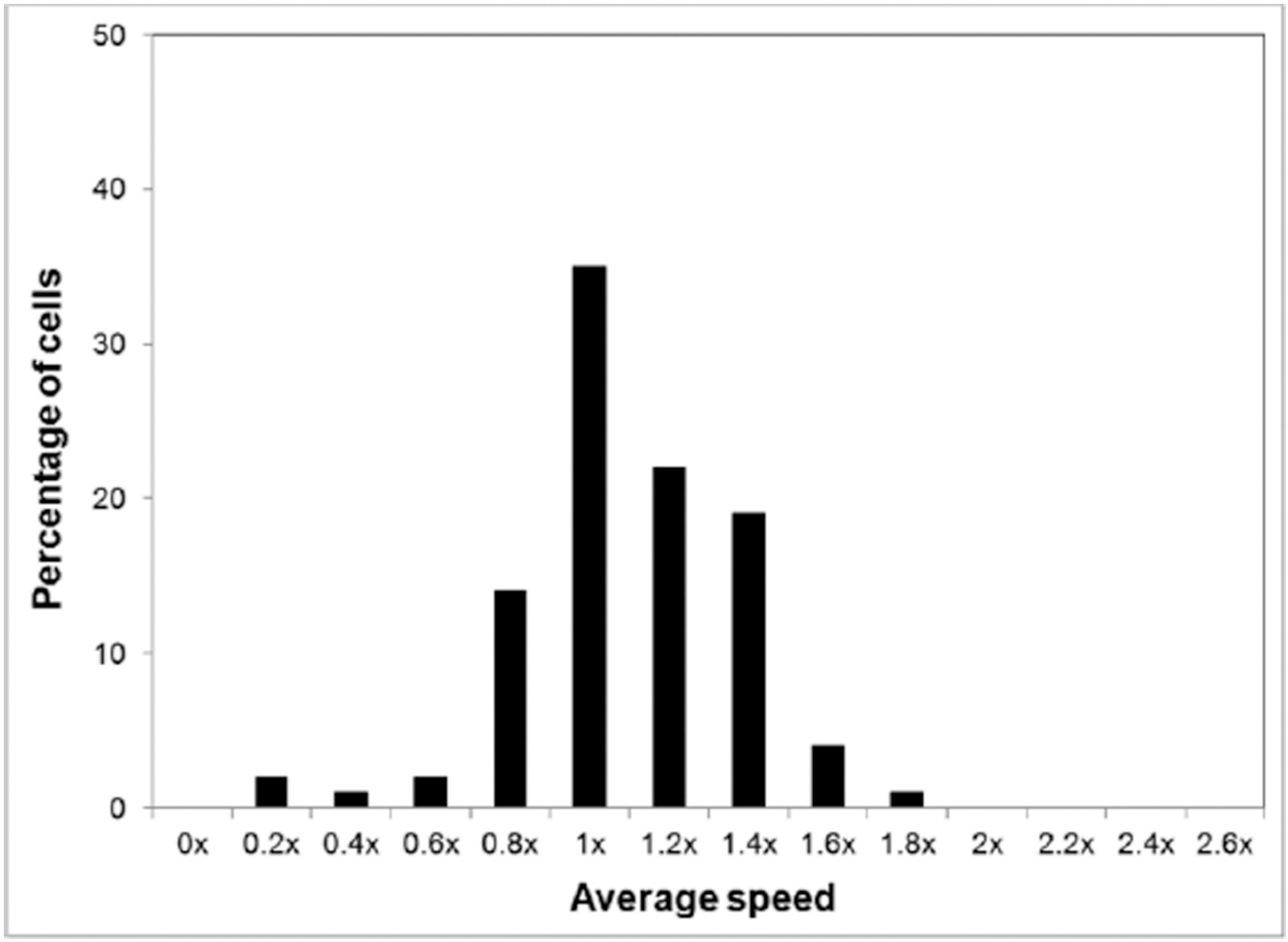

Figure 1.
Distribution of *M. penetrans* HP88 gliding speeds about the mean. Gliding speeds of the individual motile cells were grouped into bins of 0.2x of the mean, designated by *x*.

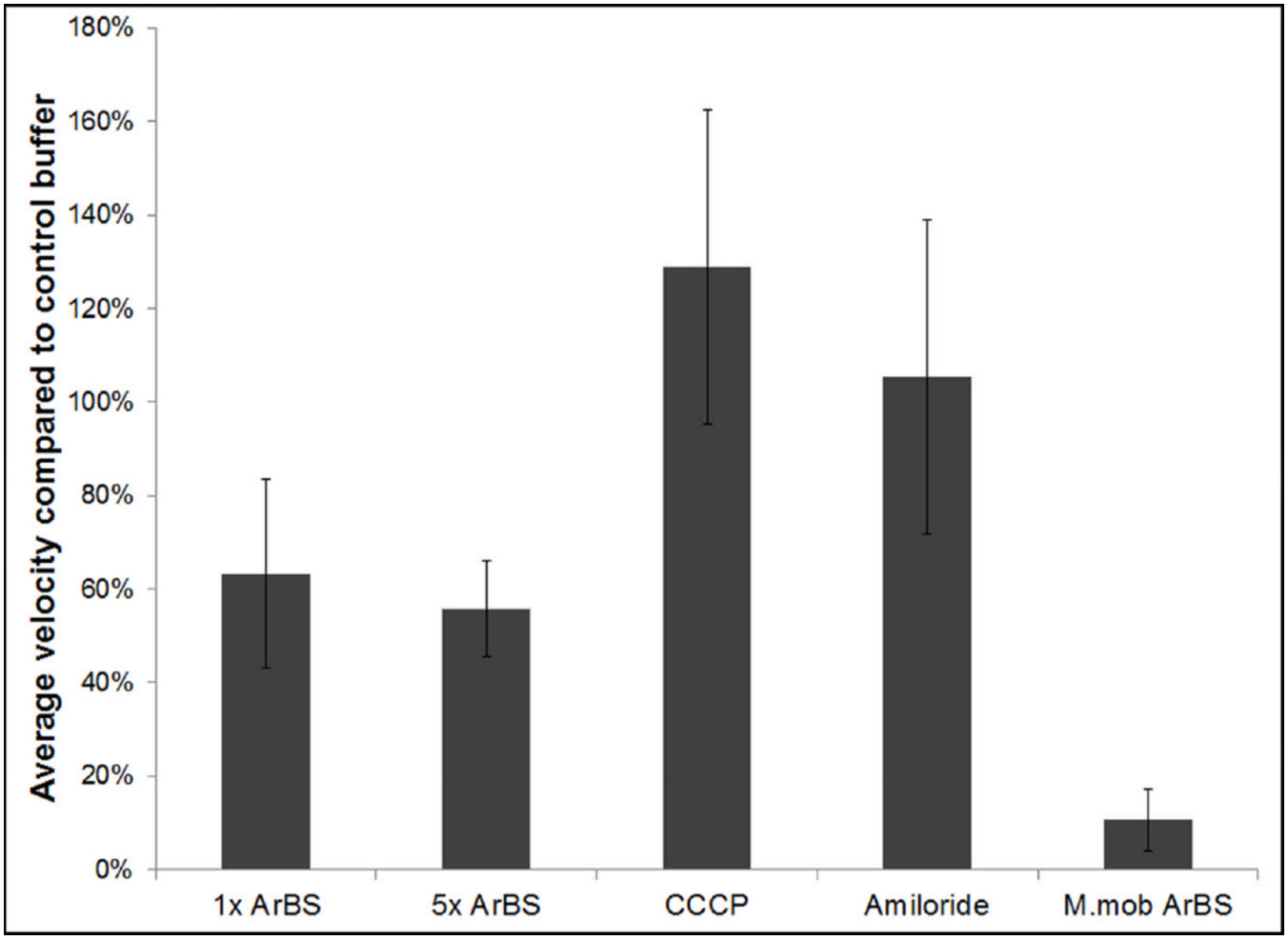

Figure 2.

M. penetrans relative gliding speed in the presence and absence of arsenate, CCCP, and amiloride compared to the average gliding speed of *M. mobile* in the presence of 50 mM arsenate. Values were normalized to respective control buffers. 1x ArBS, arsenate-buffered saline with 50 mM arsenate; 5x ArBS, arsenate-buffered saline with 250 mM arsenate.

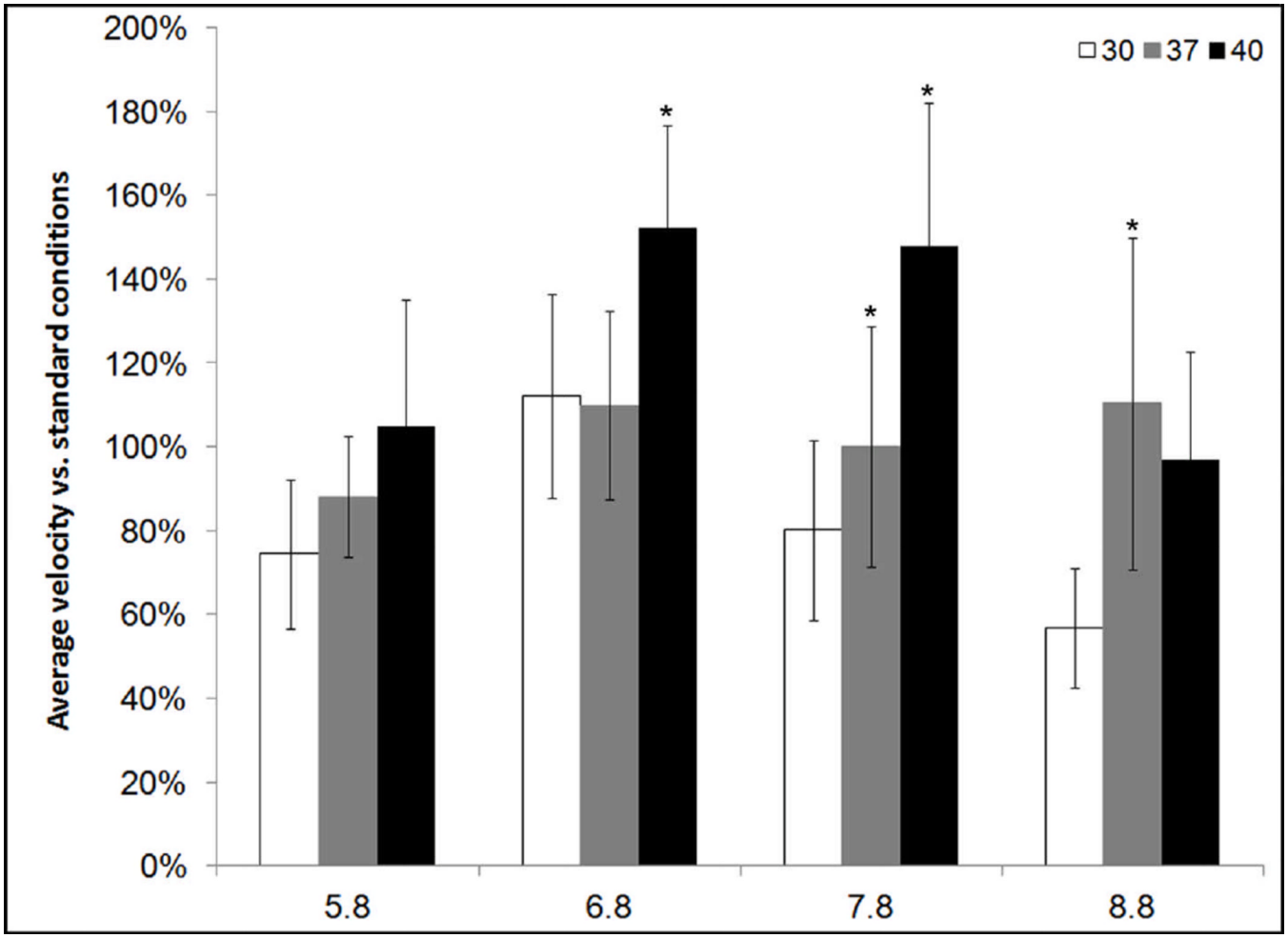

Figure 3.

M. penetrans relative gliding speed under conditions of varying temperature and pH. Values were normalized to mean gliding speed at 37°C and pH 7.8. Asterisks indicate significant differences from the next lower temperature at the same pH ($p<0.0001$) as determined by two-factor factorial analysis of variance.

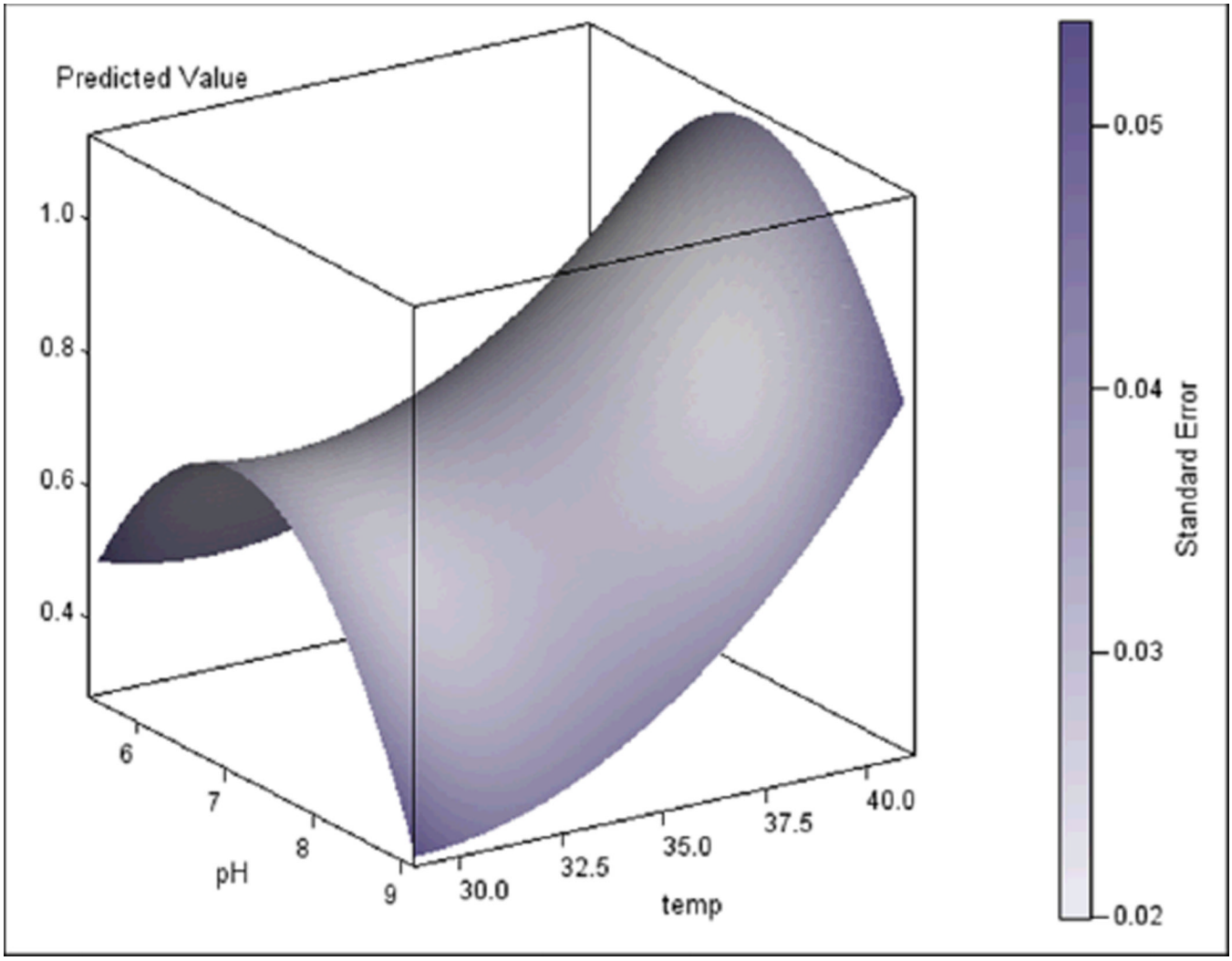

Figure 4.
Fitted response surface of *M. penetrans* motility generated by the temperature and pH motility experiment.