The motile and tactic behaviour of *Pseudomonas aeruginosa* in anaerobic environments

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ATP generated by the anaerobic metabolism of L-arginine in *Pseudomonas aeruginosa* was used to maintain the membrane potential. Although both the ATP concentration and membrane potential were lower than in aerobically incubated bacteria, motility and chemotaxis were almost normal. Venturicidin stopped anaerobic motility by abolishing the membrane potential. The addition of venturicidin to aerobic bacteria caused an increase in the membrane potential, but a decrease in internal ATP concentration, resulting in bacteria which were motile but non-chemotactic. The membrane potential was the only requirement for continued motility but ATP was required in addition for chemotaxis.

Pseudomonas aeruginosa   *Membrane potential*   *ATP*   *Bacterial motility*   *Flagellar rotation*

1. INTRODUCTION

Flagellate bacteria use the protonmotive force (PMF) across the cytoplasmic membrane to drive the rotation of semi-rigid helical flagella. By controlling the frequency of switching between clockwise and anticlockwise flagellar rotation, bacteria are able to bias their movement towards an optimum environment (review [1]). Bacteria with a single polar flagellum (e.g., *Pseudomonas aeruginosa*) can swim as efficiently using clockwise or anti-clockwise flagellar rotation. However, as with peritrichously flagellate bacteria, when the flagellum stops and changes the direction of rotation a new direction of swimming often results. When moving in a favourable direction the frequency of the reversals of flagellar rotation is suppressed.

The tactic signals controlling the frequency of direction-changing come from receptors located in the cytoplasmic membrane. Chemotactic signals arise from the binding of specific receptors to sensory transducing proteins, methyl-accepting chemotaxis proteins (MCPs) [2]. Aerotactic signals arise from the increase in membrane potential caused by an active respiratory electron-transport system [3]. The intracellular signal produced by these two different receptor systems is unknown.

Observations with *Salmonella*, *Bacillus subtilis* and *Escherichia coli* have suggested that at low membrane potentials taxis is suppressed [4]. However, there is also evidence that ATP is essential [5]. We have now investigated a system where ATP and PMF are clearly linked and controllable.

P. aeruginosa is an obligate aerobe which becomes non-motile as the PMF collapses in an anaerobic environment. Motility but not growth can be maintained anaerobically by the addition of L-arginine [6]. We have investigated the mechanism by which L-arginine can sustain motility in anaerobically incubated *P. aeruginosa*, measured the approximate membrane potential and ATP concentration which can be maintained, and examined the chemotactic and aerotactic response of the bacteria in aerobic and anaerobic environments.

We show that ATP produced by anaerobic L-arginine metabolism was used to maintain the

PMF via a venturicidin-sensitive site, presumably a reversed ATP synthetase. Except for variations in velocity there were no major observable behavioural differences between *P. aeruginosa* swimming aerobically using respiratory-generated PMF and those using arginine anaerobically. In *P. aeruginosa* a critical concentration of ATP was required for chemotactic responses but the PMF was the only requirement for continued motility. Aerobic bacteria incubated with venturicidin had very high membrane potentials but low ATP levels and, although very motile, they showed no chemotactic response.

2. MATERIALS AND METHODS

2.1. *Organism and growth*

Pseudomonas aeruginosa PAC1 was used throughout this study. Cultures were grown with vigorous shaking to exponential phase in Nutrient Broth no.2 (Oxoid) at 37°C. The bacteria were isolated into 1 mM potassium phosphate buffer (pH 7.2) containing 10 μM sodium EDTA and 100 μM Mg^{2+}, after washing twice with the same buffer.

The cultures were divided into two parts, one kept aerobic by shaking or bubbling with air, the other bubbled with oxygen-free nitrogen.

2.2. *Motility studies*

After the addition of the inhibitor or metabolite under investigation, samples were drawn into optically flat capillary tubes (Camlab) and sealed. Microscopic investigation started about 5 s after sealing the capillary tubes. Controls used buffer or ethanol, minus the metabolite or inhibitor. The behaviour of the bacteria was videotaped and later analysed for direction-changing frequency and velocity.

2.3. *Chemotaxis and aerotaxis*

Microscopic examination showed that *P. aeruginosa* in oxygen-saturated buffer, or in anaerobic buffer supplemented with 1 mM L-arginine, could remain maximally motile for about 60 min, anaerobic bacteria in unsupplemented buffer became non-motile within 5 min of transfer. The brief period of motility was probably the result of slight aeration, inevitable during experimental manipulation.

Tactic responses to gradients were measured using the chemotactic chambers described in [7]. Bacteria (0.2 ml) in either oxygenated or oxygen-free buffer were added to the blind chamber of the chemotaxis wells, a polycarbonate membrane (8 μm pore size) sealed over the chamber and the upper chamber filled with oxygen-free or aerobic buffer with or without attractant. The units were sealed and left for 10 min to allow accidentally introduced oxygen to be consumed and then the wells inverted. The number of bacteria passing through the polycarbonate membrane into the upper chamber was measured by removing samples (0.1 ml) from the top chamber and counting on a Coulter counter (model ZF) after 60 min incubation at 30°C.

2.4. *Measurement of membrane potential*

The electrical component of the PMF was measured using the distribution of $[^3H]TPP^+$ (tetraphenyl phosphonium bromide) across the cytoplasmic membrane as in [4]. Samples were incubated aerobically or anaerobically with 1.25 μCi/ml $[^3H]TPP^+$. Samples (50 μl) were withdrawn at 2 min intervals and added to 2 ml ice-cold oxygen-free buffer and filtered onto 0.6 μm pore polycarbonate membranes under either nitrogen or air. After washing with a further 2 ml ice-cold buffer and air drying, the amount of radioactivity on the membrane was counted and the membrane potential calculated, using the Nernst equation, by comparison with the extracellular radioactivity. Heat-killed and filtered bacteria were used to calculate the radioactivity bound to the cell wall and filter membrane.

The internal cell volume was measured by the dextran blue exclusion assay [8].

2.5. *ATP measurement*

The ATP content of the bacteria under different conditions was measured by grinding bacteria frozen in liquid nitrogen with an equal volume of trichloroacetic acid–ethanol (20:40%). This ensured that after thawing naturally the bacteria were in intimate contact with the trichloroacetic acid at temperatures below −10°C for at least 15 min. After removal of the trichloroacetic acid with 5 extractions with diethyl ether the ATP content was measured by a constant light signal luciferase assay (Boehringer) sensitive to 2.5 × 10^{-11} M.

Table 1

Difference in motile behaviour, membrane potential and ATP of *P. aeruginosa* incubated aerobically and anaerobically with L-arginine (1 mM)

Conditions	Velocity[a] (μm/s)		Direction-[a] changing (s^{-1})	Membrane ($\Delta\Psi$) potential (mV)	ATP (mM)
	Av.	Max.			
Aerobic					
1 min	88	120	0.8	147	4.4
50 min	70				3.8
60 min	33			97	
70 min	17			77	
75 min	6			44	0.5
Aerobic +					
venturicidin		220	0.4	160	0.8
Anaerobic	0			0	0.4
Anaerobic +					
L-arginine					
1 min	56	109	0.6	91	1.3
45 min	48				1.6
50 min	17		0.2		
60 min	5		0.2		0.3
Anaerobic +					
L-arginine +					
venturicidin					
1 min	62	188		122	2.0
2.5 min	30			70	0.8
3.5 min	0			0	0.2

[a] Average of 50 bacteria

3. RESULTS

3.1. L-arginine and motility

L-arginine is metabolised anaerobically to produce ATP by substrate-level phosphorylation via a citrulline-ornithine pathway and results in synthesis of 1 ATP/L-arginine metabolised [6].

Table 1 shows that *P. aeruginosa* incubated anaerobically in oxygen-free buffer became non-motile within 5 min, but the presence of oxygen-free L-arginine (1 mM) allowed motility to continue for up to 60 min. Bacteria incubated in aerobic buffer remained motile as long as oxygen was present.

The addition of venturicidin (10 μg/ml), a specific ATP synthetase inhibitor, to aerobically incubated *P. aeruginosa* caused an increase in the speed of translational swimming but had little effect on the length of time the bacteria remained motile. There was however a decrease in direction-changing frequency. When the same concentration of venturicidin was added to anaerobic bacteria supplemented with 1 mM L-arginine motility stopped within 3 min, although untreated bacteria remained motile for about 60 min. The role of electron transport could not be directly measured as intact *P. aeruginosa* were resistant to antimycin A and other inhibitors of electron transport.

3.2. Membrane potential measurements

The accurate measurement of membrane potentials using the distribution of permeant ions is controversial. However, in this study we were interested in a comparison of the membrane potential of aerobically incubated and anaerobically incubated bacteria supplemented with arginine. Thus, although the absolute value of the mem-

brane potential may not be accurate, it gives a useful comparative measure of the membrane potential under different conditions.

Anaerobically incubated *P. aeruginosa* had no measurable membrane potential but the addition of L-arginine caused a rise of membrane potential within 2 min to about 80 mV ± 20 mV. Aerobic *P. aeruginosa* had a membrane potential of about 140 mV ± 10 mV, which after anaerobic incubation for about 60 min fell steadily over about 15 min to about 40 mV, then rapidly over about 5 min to zero. The addition of venturicidin to aerobic bacteria causes an increase in membrane potential, in some instances to 240 mV whereas its addition to anaerobically incubated bacteria with arginine caused a brief rise and then a fall in the membrane potential.

The lower membrane potential in anaerobic, arginine-treated bacteria compared to aerobically incubated bacteria caused a difference in the speed of motility. In capillary tubes aerobic bacteria moved on average at 88 μm/s, changing direction about twice every second. Anaerobic, arginine-treated bacteria sealed into capillary tubes swam with a reduced speed of about 56 μm/s with less than two direction changes/s, and the speed was maintained for a time, dependent on the concentration of arginine. When the aerobic culture became anaerobic or the anaerobic culture exhausted the available arginine there was a period of about 5 min when the bacteria individually started to slow and then stop. There was a period of about 3 min when the bacteria moved very slowly (<10 μm/s) and direction-changing appeared suppressed, just before stopping there was a brief uncoordinated period of 'twitching'.

3.3. Intracellular ATP concentrations

The ATP content of untreated *P. aeruginosa* was about 4 mM, falling rapidly as the bacteria became anaerobic (table 1). The addition of venturicidin to aerobic *P. aeruginosa*, while having little effect on the membrane potential or bacterial motility, reduced the ATP level to about 0.8 mM. This was the result of the inhibition of ATP synthetase, the remaining ATP formation presumably being the result of residual substrate level phosphorylation.

Bacteria incubated anaerobically with arginine maintained a steady internal level of ATP, about 1.3 mM, until the arginine was exhausted. The addition of venturicidin to anaerobic, arginine-treated suspensions resulted in a brief increase in ATP to about 2 mM, presumably because the ATP-generating citrulline pathway continued but the ATP was no longer hydrolysed to maintain the membrane potential.

3.4. Chemotaxis and aerotaxis

Pseudomonas aeruginosa showed chemotaxis to L-serine [9] (table 2). Bacteria suspended in aerobic buffer showed positive chemotaxis to L-serine in aerobic buffer while anaerobic bacteria incubated with arginine suspended in oxygen-free buffer showed positive chemotaxis to L-serine in oxygen-free buffer. The maximum response being

Table 2

Chemotactic and aerotactic response of aerobic and anaerobic *P. aeruginosa*

Lower well	Upper well[a]			
	O₂-free buffer	O₂-free buffer + 0.1 mM L-serine	O₂ buffer	O₂ buffer + 0.1 mM L-serine
Anaerobic bacteria + 1 mM L-arginine	0.5	1.2	1.3	2.6
Aerobic bacteria	1.2	1.5	1.9	3.6
Aerobic bacteria + venturicidin				0.5

[a] Number of bacteria ($\times 10^7$) passing through membrane into upper bacteria-free well in 60 min as described in section 2

elicited by 0.1 mM L-serine. In order to obtain the maximum response, the number of bacteria passing through the membrane into the cell free chamber was measured after 60 min. The chemotactic response of anaerobically incubated bacteria to 10 mM L-serine was greatly increased if the L-serine was in oxygenated buffer. When venturicidin was added to the aerobic bacteria, motility continued at a maximum rate. However, the bacteria showed no chemotactic response to L-serine.

4. DISCUSSION

There is good evidence that the PMF, not ATP, is the direct energy source for flagellar rotation, although ATP is probably required for tactic responses [5]. The results presented here support this conclusion. *P. aeruginosa* incubated in conditions which allowed the formation of a PMF, but not ATP, remained motile, but not chemotactic, whereas conditions allowing ATP synthesis but inhibiting the PMF resulted in loss of motility.

If flagellar rotation in *P. aeruginosa* relied on the PMF across the membrane, as seems to be the case in all bacteria studied so far, motility exhibited by the bacteria incubated anaerobically with arginine would rely on the ATP generated anaerobically by the citrulline-ornithine pathway to maintain that PMF. Venturicidin (unlike oligomycin) is able to penetrate the outer membrane of most Gram negative bacteria and appears to bind specifically to the F_o component of the bacterial ATPase, inhibiting proton translocation across the cell membrane via this route [8]. The addition of venturicidin to aerobically incubated *P. aeruginosa* had no effect on the duration of motility, but caused an increase in swimming speed, continued respiration causing an increase in the PMF. As expected, the intracellular ATP concentration fell, confirming the inhibition of the ATPase. The PMF appeared therefore to be the only requirement for motility in aerobically incubated bacteria.

P. aeruginosa was non-motile when incubated in oxygen-free buffer. The addition of L-arginine however, allowed motility to continue, for times relating directly to the amount of L-arginine added. The addition of venturicidin to these bacteria

inhibited motility within 3 min. The supply of energy for flagellar rotation in anaerobically incubated *P. aeruginosa* must therefore rely on a venturicidin-sensitive site. The membrane-bound, venturicidin-sensitive ATPase is probably used as an ATP hydrolase to maintain the PMF in anaerobic environments using the ATP generated from arginine metabolism.

The membrane potential component of the PMF generated by ATP hydrolysis however, was higher than the suggested baseline for taxis [4], and remained at that level until just before the L-arginine supply was exhausted. This membrane potential allowed reduced motility and normal tactic responses.

Comparison of the tactic behaviour of aerobically incubated bacteria and anaerobically incubated bacteria showed that even if a very high membrane potential was maintained, the reduction of the intracellular ATP by the addition of venturicidin to the aerobic bacteria caused the loss of the ability to respond to a stimulus. Inhibited bacteria exhibited some direction-changing but at a reduced level. Direction-changing only stopped completely just before motility ceased in both anaerobic and aerobic bacteria. Although an unknown secondary effect of venturicidin on motility cannot be excluded, these results suggest that a higher concentration of ATP is required to allow changes in the tumbling rate in response to a stimulus than the concentration required in *P. aeruginosa* for unstimulated direction-changing. The critical concentration required to allow a response to a tactic stimulus appeared to be 0.8–1.3 nM ATP.

The involvement of ATP in the direction-changing response of bacteria suggests, in agreement with results obtained with *Rhodopseudomonas sphaeroides*, that the control of the direction of rotation of the flagellar motor may involve phosphorylation.

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