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Membrane potential, proton and sodium motive forces in *Azospirillum brasilense* Sp7-S

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Abstract

Some biophysical features of the diazotroph *Azospirillum brasilense* Sp7-S were studied. It maintained a stable but relatively low membrane potential ($\Delta\Psi$) of about −85 mV in conditions typical of aerated soil solutions. Internal pH was also well regulated at about 7.4 from pH 6–8. The calculated proton motive force was maximal at −220 mV in pH 5.0 medium, decreasing to zero at pH 8.8. This proton motive force appears to be insufficient to drive metabolic processes such as cell growth and flagellar rotation in neutral and alkaline media. These results prompted a study of the sodium motive force. Intracellular sodium is kept very low relative to the medium, generating a large sodium motive force of about −180 mV at pH 7. Furthermore, *A. brasilense* motility was sensitive to amiloride and benzamil, inhibitors of Na^+-driven flagellar motors. We propose that a sodium motive force can be generated and utilised for metabolic processes in *A. brasilense*. These chemiosmotic parameters may explain both the poor distribution of *Azospirillum* in acid soils and their requirement for sodium in alkaline environments.

Keywords: *Azospirillum*; Membrane potential; Ion motive force; Flagella rotation

1. Introduction

Diazotrophic azospirilla associate with the roots of agronomically important cereals such as wheat. Currently, there is interest in improving the nitrogen nutrition of the plant by the transfer of newly fixed bacterial nitrogen, which has both economic and environmental advantages. Some relevant issues in this research are the survival of azospirilla in various soil environments, their colonisation of plant roots and their ability to retain ammonia as free-living diazotrophs [1–3]. These activities rely on the bioenergetic

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Abbreviations: $\Delta\Psi$, membrane potential from inside cell to outside; ΔpH, pH gradient from inside cell to environment; PMF, proton motive force from the environment to inside the cell; Bis-tris-propane, (1,3-bis[tris(hydroxymethyl)methylamino]propane; DMO, 5,5-dimethyl-2,4-oxazolidinedione; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MES, 2-[*N*-morpholino]ethanesulfonic acid; NaMF, sodium motive force from environment to inside the cell

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status of the cell [4–6]. Therefore, knowledge of the bioenergetic status of azospirilla is necessary for a more complete understanding of the bacterial-plant interaction.

According to the chemiosmotic theory, cells store energy generated during respiration by forming both a charge gradient (membrane potential, $\Delta\Psi$) and a chemical gradient across a membrane system. This chemical gradient can be formed by the active extrusion of protons (hence a pH differential; ΔpH) or sodium ions. Metabolic activities, such as ATP synthesis, flagella rotation and ammonium accumulation, can be coupled to the dissipation of these gradients as the driving ion (H^+ or Na^+) is allowed to flow through specific membrane-bound channels such the ATP synthases, flagella motors and ammonium permeases.

Despite the importance of these biophysical parameters, they are difficult to quantify accurately. To simplify these measurements in azospirilla only a narrow range of physiological conditions likely to be encountered in agricultural soils were studied. From this approach we seek to make reasonable predictions on the biophysical status of this bacterial genus in its natural environment [7].

This paper presents data on the membrane potential ($\Delta\Psi$), internal pH and proton motive force (PMF) for *Azospirillum brasilense* Sp7-S. The PMF alone cannot explain azospirilla's metabolism at neutral and alkaline pH. We have found that a sodium motive force (NaMF) can be generated and utilised by this *Azospirillum* to drive metabolic activities under certain soil conditions.

2. Materials and methods

2.1. Bacterial strains and media

A. brasilense Sp7-S was used because of its non-flocculating phenotype and lack of exopolysaccharides [8]. Basal growth medium (NSM) was based on ionic concentrations in excluded soil solutions [9] and consisted of 10 mM DL-malate (free acid), 0.5 mM K_2HPO_4, 0.81 mM $MgSO_4$, 0.134 mM $CaCl_2$, 23 μM Fe^{3+}-EDTA, 5 mM MES buffer, 10 mM NH_4Cl, trace elements [8] at pH 6.0 adjusted with NaOH (30 mM final concentration). For assays at pH 7 or above, MES was replaced with bis-tris-propane. Cultures were shaken or sparged with air to maintain aerobic metabolism. Resazurin was used in duplicates to check the aerobic state of cells during experiments. Cell growth and experiments were conducted at 25°C with absorbance measured at 600 nm on a Cary spectrophotometer. Amiloride and benzamil were purchased from Sigma and dissolved in dimethyl sulfoxide. *Escherichia coli* S17.1 was grown in Luria-Bertani broth [8].

2.2. Cell volume and internal pH determination

When volatile compounds were assayed, cells were separated from bulk media using silicone oil centrifugation techniques [10,11]. Cell volume was empirically measured using tritiated water, and tritiated inulin to correct for the extracellular volume in the cell pellet. [^{14}C]5,5-Dimethyl-2,4-oxazolidinedione (DMO; pK_a 6.32) was used to assay intracellular pH up to external pH of 8.0. The accumulation ratio of the charged species was then applied to the Henderson-Hasselbach equation [10].

2.3. Intracellular potassium

Cells were filtered through 0.45-μm polycarbonate discs using an isotonic $Ca(NO_3)_2$ wash solution and digested in 1 M HNO_3 at 90°C for 60 min. Digest and culture media were analysed for K^+ by atomic absorption spectroscopy (AAS; Spectr20 AA, Varian). All glassware was acid washed followed by nanopure water.

2.4. Membrane potential

The uptake and equilibration of $^{86}Rb^+$ was used to calculate the membrane potential according to the Nernst equation (see Eq. 1 below). Cells were filtered on pre-washed 0.45-μm polycarbonate discs, with extracellular label removed by washing with unlabelled basal medium and discs added to scintillant for counting [10]. At time zero ($\sim$5 s) and after the washing procedure ($\sim$30 s) there was no bound extracellular radioisotope.

2.5. Intracellular sodium

^{22}Sodium^{+} was used to determine the intracellular Na^{+} concentration [12]. Cells were allowed to equilibrate with $^{22}Na^{+}$ in NSM (pH 7) for 30 min. Cells were filtered onto 0.45-μm polycarbonate discs, which were washed carefully with 3 volumes of isotonic $Ca(NO_3)_2$ and added to scintillant for counting.

2.6. Growth rates

Cells grown overnight in NSM were centrifuged and washed in fresh NSM at the appropriate pH. The growth of cultures was measured by absorbance at 600 nm every 30 min. Medium pH was monitored and kept constant as necessary.

2.7. Flagella rotation

Cells were qualitatively assessed for swimming speed in liquid cultures by phase contrast microscopy. Observations of well aerated cultures were made in a Thomas Scientific cell counter (40 μl deep) under a cover slip and at medium magnification.

2.8. Statistical treatment and calculations

All data reported are means with 95% confidence intervals and the degrees of freedom (df) indicated. Careful consideration to the propagation of errors was given throughout experiments and the subsequent calculations [13]. The Nernst criterion was used to calculate the ionic force and to identify active transport of ions across a membrane system at 25°C: at electrochemical equilibrium (no net driving force), the Nernst equation is:

$$\Delta\Psi_{i,o} = -\frac{59}{z}\log_{10}\frac{[J^z]_i}{[J^z]_o} \quad (1)$$

where J is an equilibrated ion with valency z, i is inside cytoplasm and o is outside. The net electromotive driving force (PMF or NaMF) can be calculated from the difference between the left and right hand sides of Eq. 1.

3. Results

3.1. Cell volume

In our experimental procedure 1 ml of *A. brasilense* Sp7-S culture at $OD_{600nm} = 1.0$ had an internal cell volume of $1.23 \pm 0.10 \times 10^{-9}$ m^3 (50 df) Similarly, the extracellular volume of the pellet during silicon oil separation was measured as $0.63 \pm 0.07 \times 10^{-9}$ m^3 (50 df). These volumes are a function of our methods and growth media and were used in the subsequent

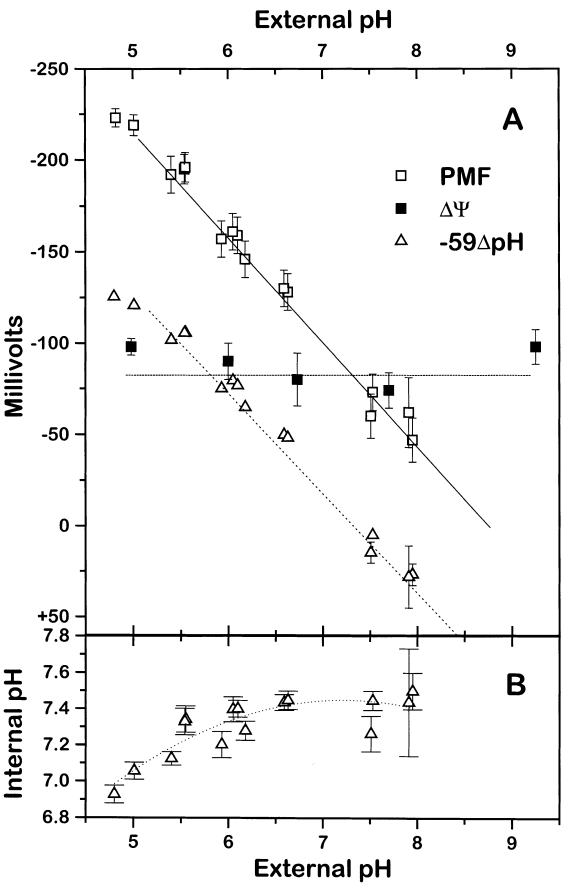

Fig. 1. Effects of external pH on ΔΨ, internal pH and PMF in *A. brasilense* Sp7-S. ΔΨ and internal pH were measured by equilibrium accumulation of $^{86}Rb^{+}$ and ^{14}C-DMO, respectively. All data are means ± the 95% confidence interval calculated using at least 5 df. Error bars were omitted when smaller than the symbol. Lines of best fit are displayed. A: Data presented in mV according to Eq. 1. ΔΨ, closed squares; ΔpH, open triangles; PMF, open squares. B: Internal pH, open triangles.

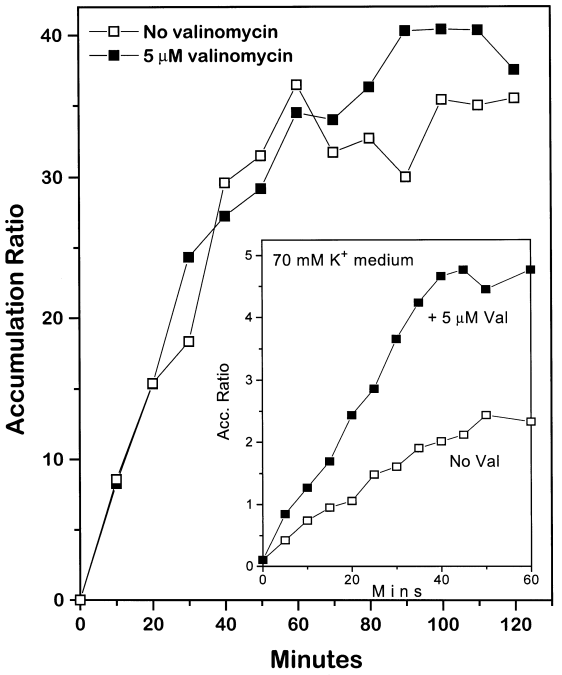

Fig. 2. Accumulation of $^{86}Rb^{+}$ by *A. brasilense* Sp7-S over time in 1 mM K^{+} NSM (main) and 70 mM K^{+} NSM (inset). Closed squares with 5 μM valinomycin; open squares without.

calculations of internal ionic concentrations and external binding of radioisotopes as required.

3.2. Intracellular potassium

In NSM, the intracellular K^{+} levels in Sp7-S were 152 ± 20 mM (15 df). This value was used in calculations of activity coefficients (see Section 3.4 and Fig. 3).

3.3. Intracellular pH

A. brasilense Sp7-S displayed an internal pH of about 7.4 (70 df) in neutral pH medium (Fig. 1A,B). Internal pH fell to about 6.8 as the external pH was moved to pH 5. Within 10 min the inhibitors FCCP (10 μM) and DNP (1 mM) collapsed the pH gradient (data not shown).

3.4. Membrane potential

In NSM, containing 1 mM K^{+}, $^{86}Rb^{+}$ equilibration occurred after 120 min (Fig. 2). Valinomycin, a K^{+}/Rb^{+} ionophore, did not affect accumulation rates or equilibration levels in 1 mM K^{+} medium (Fig. 2, main). However, for cells grown in high K^{+} medium, valinomycin functioned to equilibrate $^{86}Rb^{+}$ according to the ΔΨ rather than with the K^{+} potential (Fig. 2, inset). Valinomycin was retained for all ΔΨ experiments. The ΔΨ was calculated using equilibrium accumulation ratios of $^{86}Rb^{+}$ in valinomycin-treated cells (Eq. 1). The ΔΨ was maintained at about −85 mV between pH_o 5 and 9.25 (Fig. 1A). The ΔΨ was depolarised by logarithmic increases in external K^{+} concentrations, 10 μM FCCP, 1 mM DNP or external pH 4.5 (Fig. 3). A previous study of *A. brasilense* ΔΨ used EDTA to permeabilise cells to a lipophilic cation probe [14]. A

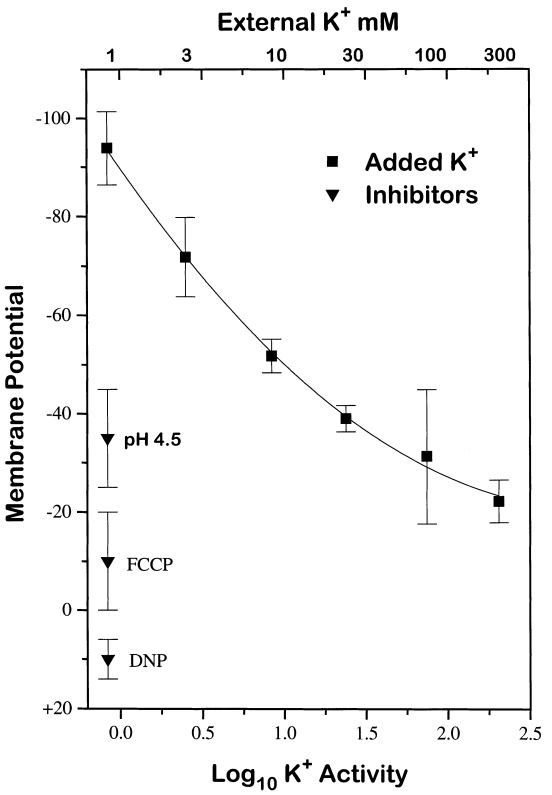

Fig. 3. The effect of increased K^{+}, 10 μM FCCP, 1 mM DNP and pH 4.5 on ΔΨ of *A. brasilense* Sp7-S. Additions were made at time zero, with data presented as means ± 95% confidence intervals with 5 df. Closed squares, effect of increased K^{+} with a line of best fit shown; closed triangles, effect of various treatments in NSM at pH 6 containing 1 mM K^{+}.

similar EDTA pre-treatment did not affect *A. brasilense* Sp7-S $\Delta\Psi$ measured by $^{86}Rb^+$ accumulation.

3.5. Proton motive force

The PMF for Sp7-S over the pH range 5–8 was calculated (Eq. 1; Fig. 1A). In the absence of reliable ΔpH data at these alkaline pH values (data not shown), PMF was extrapolated to an external pH of 8.8. The PMF was greatest at -220 mV when pH_o 5, decaying at -58 mV pH unit^{-1} to 0 when external pH was 8.8.

3.6. Growth rates

In our aerobic basal growth medium, NSM, Sp7-S was able to grow well at neutral pH. At external pH 4.5, 6.0, 7.4 and 8.4 doubling times (in hours) were 13 ± 3, 3.5 ± 0.2, 3.0 ± 0.2 and 7.2 ± 0.4 (each with 16 df), respectively. There was no growth at pH 8.8. If the medium pH is not adjusted then alkalisation due to malate consumption and respiration does not continue beyond pH 8.8 (data not shown).

Amiloride and benzamil, reported to be inhibitors of Na^+-driven flagellar motors in some bacteria [15,16], were tested for their effect on cell growth at pH 7.4. Amiloride added during the exponential phase at 3 mM allowed growth to continue unaffected for 30 min, after which growth ceased. Addition of 0.5 mM benzamil immediately halted culture growth.

3.7. Intracellular sodium and the NaMF

Atomic absorption techniques were not sensitive enough to detect internal sodium levels (data not shown) but confirmed that NSM contained 30 mM Na^+ (added as NaOH). Using a sensitive $^{22}Na^+$ radioisotope method, the intracellular sodium concentration of Sp7-S was 0.84 ± 0.28 mM (18 df) in NSM at pH 7. This isotopic data demonstrates that Na^+ was actively extruded from these cells generating an inwardly directed sodium equilibration potential. Using Eq. 1, this sodium chemical potential was calculated as $+92 \pm 8.5$ mV under these conditions. The total NaMF available to *A. brasilense* Sp7-S at pH 7 in NSM therefore equals -182 ± 10 mV (Eq. 1).

3.8. Flagella rotation

Cell swimming speed was qualitatively assessed in liquid medium. *A. brasilense* Sp7-S was able to rotate its polar flagella optimally from pH_o 6.0 to 9.1 ± 0.1. Swimming speed gradually slowed as the pH rose and ceased at pH_o 9.6. Swimming was restored, following the same response curve, if the medium pH was then progressively acidified. Amiloride (3 mM) and benzamide (0.5 mM), inhibitors of Na^+-driven flagella motors [15], stopped flagella rotation at pH 7.4. In comparison, *E. coli* S17.1 was not affected by either inhibitor [16].

4. Discussion

This study characterises the bioenergetic status of *A. brasilense* Sp7-S under the simplified conditions of aerobic metabolism with N in excess. Using well established methodologies [10,12] we have demonstrated that Sp7-S maintained a relatively constant internal pH at 7.4, a $\Delta\Psi$ at about -85 mV and a relatively low and unregulated PMF. However, *A. brasilense* Sp7-S can generate and utilise a NaMF.

Measuring the cytoplasmic volume accessible to water is important for many biophysical calculations. *A. brasilense* Sp7-S, a non-flocculating strain of *A. brasilense* Sp7, with a reduced exopolysaccharide coat was chosen for this study to reduce potential experimental problems. The basal growth medium was osmotically weak, allowing for a fully turgid cell structure. Under these conditions the periplasmic space was assumed to be negligible. Transmission electron micrographs ([8]: Fig. 1e,f) show that there are no membrane invaginations and little periplasmal space in *A. brasilense*.

Over a wide range of external pH, *A. brasilense* cells were able to regulate and stabilise their internal pH. This parameter is particularly important for determining the internal ratio of charged/uncharged species of weakly dissociating ionic species, such as ammonia. When the external pH was 4.5 the $\Delta\Psi$ was significantly depolarised. We suggest that this loss of homeostasis, reflected in very long doubling times at this pH, explains the poor distribution of azospirilla in acidic soils [17,18].

The $\Delta\Psi$ in *A. brasilense* Sp7-S was also well con-

trolled but at a relatively low level. Our results confirm and extend an earlier report of the magnitude and direction of the $\Delta\Psi$ [14]. Using a $^{86}Rb^+$ accumulation methodology potentially damaging EDTA pre-treatments and corrections for extracellular binding were avoided. *Azospirillum*'s $\Delta\Psi$ was altered using ionophores, low pH and increased external potassium (Fig. 3). Zhulin et al. ([14]: Fig. 5) demonstrated that the $\Delta\Psi$ can also respond to the oxygen concentration.

An active uptake mechanism was demonstrated for K^+. In NSM, containing 1 mM K^+ (verified using AAS), Sp7-S contained 150 mM K^+. Using Eq. 1 the Nernst equilibrium potential for K^+ would be -128 mV and given that the $\Delta\Psi$ was -85 mV then the potassium would be about +40 mV from electrochemical equilibrium and so must be actively taken up the cells. This intracellular K^+ level also indicates that $^{86}Rb^+$ equilibrates with the $\Delta\Psi$ and not the potassium equilibrium. The curvilinear response of $\Delta\Psi$ to increased K^+ (Fig. 3) could be due to the uptake and use of K^+ as an osmoticant.

The relatively low and stable $\Delta\Psi$ and well controlled internal pH result in a low and unregulated PMF (Fig. 1). This meagre PMF, especially in alkaline media, raised questions over the ability of this organism to drive metabolic processes, such as ATP synthesis and flagella rotation. A conventional stoichiometry of the F_1F_0 ATP synthase is $3H^+$ per ATP synthesised [19] and as each H^+ represents -59 mV then the threshold for ATP generation should be about -177 mV. This would be equivalent in *A. brasilense* Sp7-S to growth at pH 6 or below (Fig. 1) if only the PMF were utilised. In our basal growth medium Sp7-S was able to grow at pH 7.4 and 8.4, well above the pH where the PMF was calculated to be insufficient to drive ATP synthesis by a conventional H^+-conducting F_1F_0ase. The energy threshold for flagella rotation is lower than for ATP synthesis, at about -30 to -100 mV [20]. *A. brasilense* Sp7-S was able to rotate its polar flagella up to pH 9.6, again well beyond that expected due to the PMF alone. This evidence prompted a study of the role of Na^+ in *Azospirillum* bioenergetics.

Some bacteria are able to maintain a very low internal Na^+ concentration relative to their environment and so generate a sodium chemical potential [4,5,12,21]. The flux of Na^+ ions, as driven by the NaMF, can be linked to all membrane associated processes [4], including ATP synthases and flagella rotation. This Na^+ chemical potential component was shown to be large in Sp7-S and so combined with the $\Delta\Psi$ leads to a large electrochemical potential for Na^+ (NaMF). The calculated NaMF of -180 mV in our basal medium could drive ATP synthesis by a Na^+-driven ATPase at a conventional stoichiometry of 3 Na^+/ATP. This NaMF would allow growth to continue beyond pH 6 and above. Presumably more energy is consumed maintaining homeostasis at pH 8.4, lengthening doubling times. We assume that homeostasis is lost above pH 8.8 resulting in no growth, perhaps due to cytosol alkanisation.

A. brasilense Sp7-S could also rotate their polar flagella up to pH 9.0, calculated to have insufficient PMF to drive a H^+-driven motor (Fig. 1). Under these conditions, the NaMF may be utilised to drive flagella rotation. Furthermore, this bacterium was sensitive to inhibitors of Na^+-driven flagella motors, amiloride and benzamil [15]. These inhibitors may also interfere with other Na^+ channels in this bacterium, creating an indirect effect on polar flagellar rotation. Amiloride allowed growth to continue unaffected for about 30 min, indicating that its rapid (<30 s) inhibition of flagellar rotation could have been a direct interaction on a Na^+-driven motor.

In nature, *A. brasilense* are frequently isolated from neutral and alkaline soils, usually containing sodium [18,22,23]. Some strains of azospirilla, *A. halopraeferens* [24,22] and *A. irakense* [25], isolated from sodic, alkaline soils have a requirement for sodium in their growth media. High sodium environments would increase the NaMF and may improve azospirilla growth and survival at high pH. Furthermore, sodium is added to many azospirilla growth media [26] used in isolation and physiological studies. Some reported Na^+-dependent systems have very high affinities for sodium (K_m about 20–40 μM; [21]). Low levels of Na^+ contamination may be sufficient to create a sodium cycle, often obscuring the role of sodium in metabolism. An examination of *A. brasilense*'s Na^+ dependence was not attempted in this study.

This bioenergetic study of *A. brasilense* has found that this soil-borne diazotroph can generate and utilise both H^+ and Na^+ motive forces, with their rel-

ative importance perhaps dependent on soil conditions. Variations in the magnitude and utilisation of these ionic motive forces may explain some of the different species characteristics within the *Azospirillum* genus.

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