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Behavior of *Rhizobium meliloti* in oxygen gradients

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Abstract ***Rhizobium meliloti*** **cells responded to an abrupt change in oxygen concentration by changing the cell speed (chemokinesis), but they did not alter the frequency at which swimming cells stopped briefly (aerotaxis). Changes in cell speed upon stimulation with oxygen coincided with changes in membrane potential. The cells did not form an aerotactic band in a spatial gradient of oxygen as do the cells of other bacterial species. The *fixL* and *fixJ* genes which encode a heme-containing protein kinase that senses oxygen and a response regulator, respectively, were not involved in the behavior of *R. meliloti* in oxygen gradients.**

Key words: Oxygen; Chemokinesis; Membrane potential; FixL; FixJ; *Rhizobium*

1. Introduction

In *Salmonella typhimurium* and *Escherichia coli,* the initial sensory transduction events in the behavioral response to oxygen (aerotaxis) involve modulation of the flow of reducing equivalents through the respiratory chain [1]. An unidentified cellular component senses the changes in the redox state of the constituents in the respiratory chain, and/or consequent changes in the proton motive force, and transmits these signals to the flagellar motors [2–5]. The aerotactic response in *Escherichia coli* requires the CheA chemotaxis protein, a histidine kinase, and the CheY protein, a regulator of the bacterial flagellar motor [6]. The terminal oxidases of the bacterial respiratory chain are the primary receptors for oxygen but not the transducers of the aerotactic signal [7,8]. A search continues for other membrane-bound molecules that can bind oxygen (or change their redox state) and transduce the aerotaxis signal to the flagellar motor.

One of the most interesting candidates for a sensor/transducer in the aerotactic response was the FixL protein from *Rhizobium meliloti*, a transmembrane hemoprotein with kinase activity [9]. The heme moiety on FixL binds oxygen non-cooperatively and with low affinity; consequently, it can respond linearly to an extended range of oxygen concentrations [10]. The FixL and FixJ proteins belong to a family of homologous two-component regulatory systems which consist of a histidine kinase sensor protein and a phosphorylated response regulator protein [11]. The deoxy form of the FixL kinase is active and transfers a phosphoryl group to the FixJ protein, a transcriptional activator of nitrogen fixation genes [9,12,13]. The CheA protein kinase and the CheY regulator protein in chemotaxis also constitute a two-component regulatory system. Crosstalk has been demonstrated between two-component regulatory systems in which the sensor kinase of one system can phosphorylate the response regulator protein of a different two-component regulatory system. [14]. For example, the NtrB protein kinase that regulates nitrogen metabolism can also phosphorylate the CheY protein. We investigated whether the FixL kinase has a second role as an oxygen sensor for aerotaxis that transduces the oxygen signal by cross phosphorylating the CheY protein or by another mechanism that modulates the flagellar motors. The *cheA* and *cheY* genes were recently discovered in *R. meliloti* [15].

2. Materials and methods

2.1. Bacterial strains

The *R. meliloti* strains wild type for nitrogen fixation were 102F34 and 5591 [16]. The mutant strain JC1 was derived from *R. meliloti* 102F34 by recombination with *fixL* containing a Tn*phoA* insertion and selection for kanamycin resistance [17]. The mutant *R. meliloti* 5591ΔLJ is isogenic with *R. meliloti* 5591 except for a 1323 bp internal deletion of the *fixL fixJ* region in the former. This deletion was a rare event resulting from Tn*5* mutagenesis. *R. meliloti* cells were grown in minimal medium [18], containing 2 g malate per liter as a carbon source. Aerotaxis was measured in cells growing exponentially (OD_{600} = 0.5). *Azospirillum brasilense* Sp7 (ATCC 29145) cells were grown and prepared for aerotaxis experiments as described previously [8].

2.2. Behavioral assays

The spatial assay for aerotaxis was performed as follows. A concentrated cell suspension in growth medium was sealed in an optically flat capillary of 0.2 mm path length (Vitro Dynamics Inc., Rockaway, NJ). Oxygen diffusion through the air/suspension interface and oxygen consumption by the respiring cells created a spatial gradient of dissolved oxygen. The distribution of bacteria near the interface was observed and recorded as a density profile using a microphotometer similar to one described previously [8]. Oxygen consumption by the respiring cells was measured using a Clark-type electrode. For the temporal assay of aerotaxis, bacteria were placed in a microchamber and ventilated with either oxygen or nitrogen gas as described previously [5]. The response to a change in oxygen concentration was observed and recorded through a videomicroscope. Bacterial behavior was analyzed using manual frame-by-frame analysis of the videorecordings after tracing individual tracks of free-swimming bacteria on transparent acetate sheets directly from the monitor screen [19]. Ten cells were tracked per assay, typically for 6–8 s each. Four replicates were performed on each of two different days.

2.3. Membrane potential measurements

We monitored changes in membrane potential upon stimulation of cells with oxygen using a tetraphenylphosphonium (TPP^+)-selective electrode, as previously described [7]. Cells were made permeable to TPP^+ by treatment with EDTA [7]. Non-specific binding of

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TPP^+ was determined using heat-treated cells (60°C, 30 min). Membrane potential was calculated using the Nernst equation after measuring the external TPP^+ concentration and calculating the internal TPP^+ concentration. An internal cell volume of 10^{-15} l was assumed in these calculations.

3. Results and discussion

Using the spatial assay for aerotaxis, we compared the aerotactic behavior of the wild type *Rhizobium meliloti* and mutant cells defective in the *fixL* and *fixJ* genes. Fig. 1 represents typical profiles of the distribution of *R. meliloti* cells in a spatial oxygen gradient; *A. brasilense* cells constituted a positive control for aerotaxis. An aerotactic band in the *A. brasilense* suspension formed after 1–2 min of incubation. Changes in the distribution of *R. meliloti* cells were seen only after incubation for 1 h to 2 h (growth was inhibited by the presence of 50 μg/ml chloramphenicol). Clusters of higher *R. meliloti* cell density that formed approximately 1 mm from the meniscus had less distinct borders and lower density than in the aerotactic band of *A. brasilense*. Because the respiration rate per cell in *A. brasilense* was higher than that in *R. meliloti*, the *A. brasilense* cell suspension was diluted to equalize the rate of oxygen uptake, and oxygen gradient formation in *A. brasilense* and *R. meliloti* suspensions. *R. meliloti* cells in the cluster and interior to the cluster were non-motile whereas *A. brasilense* cells were highly motile within, and on either side of, the aerotactic band. Motile *R. meliloti* cells were distributed uniformly within the zone between the cluster and the meniscus. The 21% oxygen at the meniscus neither attracted the motile cells as observed for aerobic species of bacteria [20], nor repelled the cells as observed for facultative anaerobic and microaerophilic species [7,8]. These observations indicate that *R. meliloti* cells are unable to orientate themselves in an oxygen gradient, and do not show true aerotaxis. The *fixL*, *fixJ* and wild type *R. meliloti* strains behaved similarly in spatial gradients of oxygen, indicating that neither absence nor presence of the FixL and FixJ proteins leads to the appearance of the aerotactic response.

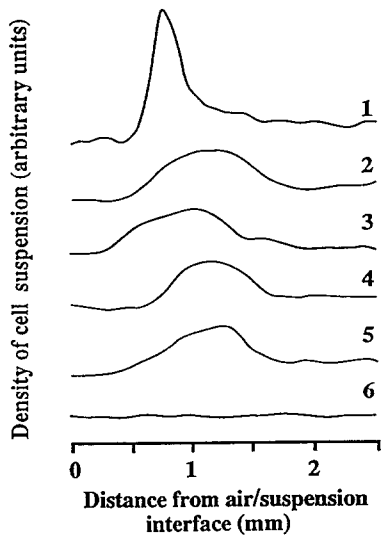

Fig. 1. Profiles of cell distribution in flat capillaries. Suspensions of *A. brasilense* Sp7 (1) (positive control), *R. meliloti* 102F34 (2), *R. meliloti* JC1 (3), *R. meliloti* 5591 (4), *R. meliloti* 5591ΔLJ (5), *R. meliloti* 102F34 plus 20 mM KCN (6) (negative control) were incubated in capillaries for 2 h, except for *A. brasilense* which was incubated for 2 min. Each capillary was scanned using a microscope equipped with a microphotometer. The optical density at 600 nm of the suspensions was 0.5 except for *A. brasilense* which was 0.3 (see text for details).

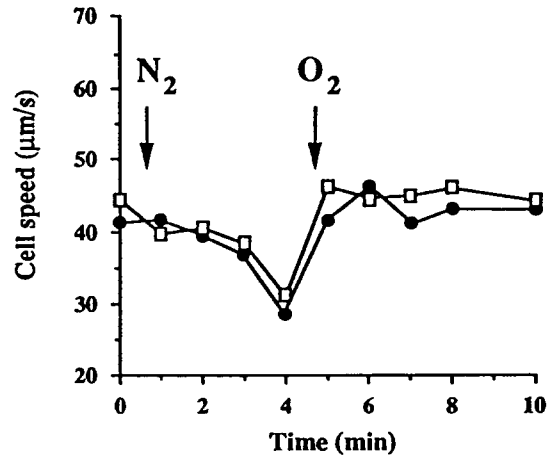

Fig. 2. Time course of the change in cell speed of free-swimming cells of *R. meliloti* after a change in the ventilating gas. A 4 μl drop of wild type cells (strain 102F34, ●) or *fixL* mutant cells (strain JC1, □) in growth medium (OD_{600} = 0.2) was placed in a microchamber on the microscope stage and the ventilating gas in the flow cell was changed to nitrogen or to 100% oxygen at the times shown by arrows. Cell speed was measured as described in section 2.

Bacteria regulate their chemotactic behavior by changing the frequency of direction changes in response to chemoeffectors. Direction changes occur when the direction of swimming is reoriented during periodic tumbling (*E. coli*), reversal (*Halobacterium salinarium*), or stopping (*R. meliloti*). In addition, chemoeffectors may cause a change in cell speed, chemokinesis [21]. The behavior of *R. meliloti* cells was quantitated in a temporal assay of aerotaxis [5]. There was no statistically significant difference in the stopping frequency in *R. meliloti* wild type or mutant cells upon abrupt exposure to either 21% or 100% of oxygen. Substitution of nitrogen for oxygen also did not change significantly the stopping frequency. This confirmed the absence of a normal aerotactic response in *R. meliloti*.

There was a chemokinetic response to oxygen, however, in wild type and in *fixL* and *fixL fixJ* mutants. Fig. 2 shows chemokinesis to oxygen in *R. meliloti* 102F34 (wild type) and in a *fixL* mutant. Similar results were obtained in strains 5591 (wild type) and 5591ΔLJ (*fixL fixJ*) (data not shown). After replacement of nitrogen by either 21% or 100% oxygen, the cell speed increased; removal of oxygen decreased the cell speed (Fig. 2). No adaptation during chemokinesis was observed. After substitution of nitrogen for air, cell speed gradually decreased and the cells stopped swimming after approximately 10 min. We anticipated that chemokinesis to oxygen was governed by the proton motive force [21]. Changes in the proton motive force (Δp) upon stimulation of the cells with oxygen or nitrogen were followed by monitoring changes in membrane potential ($\Delta\varphi$) at neutral external pH, where $\Delta\varphi$ is essentially the only component of Δp. Fig. 3 shows that $\Delta\varphi$ decreases upon addition of nitrogen and increases upon addition of oxygen. These changes coincide with changes in cell speed (Fig. 2). At an external pH of 7.5, $\Delta\varphi$ was −112 mV in cells exposed to 21% oxygen and −124 mV in cells exposed to 100% oxygen. The cell speed, however, was unchanged (Fig. 2) indicating that the

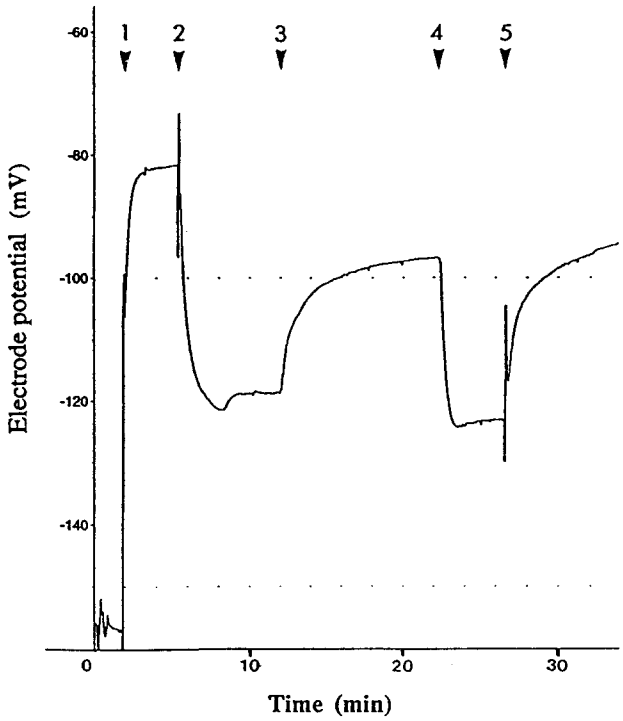

Fig. 3. Changes in membrane potential upon exposure of *R. meliloti* 102F34 cells to aerobic and anaerobic conditions. Membrane potential was monitored by a TPP^+-selective electrode as described in section 2. Nine milliliters of 10 mM HEPES (K^+) buffer (pH 7.5) containing 20 mM lactate as an energy source was placed in a vessel, equilibrated at 30°C with stirring and sparged through a needle line. The arrows indicate the times at which (1) 4 μM TPP^+ was introduced; (2) 1 ml of cells (OD_{600} ~ 20) was introduced; (3) the sparging gas was changed from 21% oxygen to nitrogen and (4) from nitrogen to 100% oxygen; (5) 5 μM valinomycin was added to collapse membrane potential.

flagellar motors have reached maximum speed at −112 mV membrane potential.

The behavior of *R. meliloti* cells in an oxygen gradient was unusual: the bacteria did not alter the frequency of stopping or migrate to a preferred concentration of oxygen. However, the cells showed a chemokinetic response to oxygen that correlated with changes in the proton motive force. The absence of true aerotaxis in *R. meliloti* and the presence of a normal aerokinetic response in *fixL* and *fixL fixJ* mutants indicates that the expressed FixL and FixJ proteins in *R. meliloti* do not have a significant regulatory role in cell behavior in oxygen gradients.

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