Aerotactic responses in bacteria to photoreleased oxygen

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Abstract

Bacterial aerotaxis is a rapid response towards or away from oxygen. Here we report on the use of computer-assisted motion analysis coupled to flash photolysis of caged oxygen to quantify aerotactic responses in bacteria. The caged compound (μ-peroxo)(μ-hydroxo)bis(bipyridyl)cobalt(III) perchlorate liberates molecular oxygen upon irradiation with near-UV light. A mixture of cells and the caged oxygen compound was placed in a capillary tube and challenged by discrete stimuli of molecular oxygen produced by photolysis. We then recorded the rate of change of direction (rcd) as an estimate of tumble frequency in response to liberated oxygen and measured the signal processing (excitation) times in Bacillus subtilis, Bacillus halodurans and Escherichia coli. This computer-assisted caged oxygen assay gives a unique physiological profile of different aerotaxis transducers in bacteria.

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1. Introduction

Aerotaxis is the migratory response towards or away from oxygen and is used by various microorganisms to swim toward an optimal oxygen concentration for their metabolism. Beginning with Engelmann’s report in 1881 that bacteria such as Bacterium termo and Spirillum tenue move to surround oxygen-producing plant cells, physiological methods have played an essential role in characterizing different aerotactic responses in motile bacteria [1]. Baracchini and Sherris [2] developed the aerotactic capillary assay and demonstrated that different microbial species form rings of different radii around air bubbles. The assay relies on oxygen consumption from bacterial respiration, which creates oxygen gradients between the open ends and the interior of the capillary. Later, a temporal assay was developed which involved step changes in the oxygen concentration within a gas perfusion chamber [11]. However, inherent limitations in the architecture of the perfusion system precluded rapid kinetic measurements of the excitation phase.

Aerotaxis sensors in Archaea and Bacteria include the heme-containing myoglobin-like HemAT transducers in Halobacterium salinarium and Bacillus subtilis [5–7], FAD-containing Aer transducers in Escherichia coli [3,4,8] and Pseudomonas putida [9], and the Tsr chemoreceptor in E. coli [4]. The HemAT transducers bind oxygen and are therefore true oxygen sensors. Capillary assays showed aerotactic band formation in wild-type B. subtilis cells and in methyl-accepting chemotaxis proteins (MCP) minus cells overexpressing hemAT-Bs from a plasmid, but not in cells deleted for all 10 putative MCP-like transducers [5]. In HemAT-Bs, a 176-residue N-terminal domain is responsible for binding heme and sensing oxygen [6], with His123 binding to the heme group [7].

In contrast to HemAT, the aerotaxis sensor Aer in E. coli does not bind oxygen directly, but likely infers the

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2. Materials and methods

2.1. Bacterial strains and growth conditions

*B. subtilis* O11085 (wild-type), O13545 Δten (all ten transducers deleted), O13555 Δten+hemAT-Bs (overexpression of HemAT-Bs), and Δten+hemAT-BsH123A (overexpression of HemAT-BsH123A) were grown in nutrient broth for 48 h or until sporulation. Spore morphology was inspected under the microscope, after which spores were washed with HPLC-grade water and frozen with liquid nitrogen. Motile cells were obtained by growing frozen spores in Horikoshi II medium to OD600 = 1.5 in a shaker at 37°C.

Two mg of (μ-peroxo)(μ-hydroxobis(bipyridyl)cobalt(III)) perchlorate, ε = 4900 M⁻¹ cm⁻¹ (Molecular Probes, Eugene, OR, USA), was dissolved in 500 μl of 50 mM potassium phosphate buffer (pH 7.4), diluted to 13.1 mM, frozen with liquid nitrogen and stored at −70°C until further use (during initial studies we used caged oxygen compound synthesized according to MacArthur et al. [15]).

2.2. Preparation of caged oxygen

Caged oxygen compound (4.37 mM final concentration) was added to *B. subtilis* and *E. coli* cells in LB media and to *B. halodurans* in Horikoshi II media (OD600 = 0.35). Flat capillary tubes (0.1×1.0 mm, #5010, VitroCom, NJ, USA) were then half-filled with the bacterial suspension/caged oxygen mixture. The capillaries were sealed at both ends with Critoseal (Monject, Sci. Division, MO, USA), placed on the microscope stage, and observed with a 40× glass phase-contrast objective. A green interference filter was interposed between the sample and light source to enhance video contrast and facilitate tracking of motile cells. Data collection occurred 30 to 45 min after preparation of the capillary tubes. Duration of the near-UV light flash was 900 ms for *B. subtilis* and 100 ms for *E. coli* and *B. halodurans*. Images of swimming bacteria were captured with a CCD camera and digitized with a VP110 video digitizer (VP120 digitizer, Expert Vision
2D/AT Release 3.1 version software; Motion Analysis, Santa Rosa, CA, USA). The digitizer was equipped with a −5 V event tone marker that signals the opening of the shutter (UniBlitz, Vincent Associates, Rochester, NJ, USA) with a 5 s delay. A custom-made NOT gate circuit connected to a Grass stimulator was interposed between the digitizer and shutter driver/timer (UniBlitz, model T132) to convert the event tone to a +35 V pulse needed to activate the shutter system. Illumination from a 100-W mercury short arc lamp (HBO 100 W/2, Osram, Munich, Germany) was passed through the electronic shutter and filtered through a Nikon fluorescence cube (360 ± 20 nm excitation, > 400 nm barrier filter, 400 nm dichroic mirror). Intensity of the UV light, measured with a silicon photodiode (XRL340B #5348, International Light, Newburyport, MA, USA), was 1.60 × 10^−4 W cm⁻². The amount of oxygen released from 4.37 mM caged oxygen was estimated to be approximately 3.27 × 10⁻¹¹ mol (0.046 pmol) for a 900-ms flash and 3.63 × 10⁻¹⁵ mol (0.0051 pmol) for a 100-ms flash (calculations not shown) using a quantum yield of 0.1 [16].

When flash photolysis was performed immediately after bacteria were introduced into the capillary tube, wild-type B. subtilis failed to respond because receptors were saturated with oxygen. A 30–60 min incubation period was used to assure a semi-anaerobic environment. A field of view (approximately 150 × 150 μm) containing 20–40 cells with a rate of change of direction (rcd) = 700–800° s⁻¹ for B. subtilis and 700–1200° s⁻¹ for E. coli was selected for observation. Data collection began 5 s before the flash, and an average of 1000 paths (30 repetitive assays) were recorded for 30 s. A stage micrometer was used to calibrate image measurements (scale factor = 0.795 μm/pixel). Although UV light acts as a repellent, it does not prevent a positive response to oxygen to occur.

2.4. Agar plug assay and effect of 360 ± 20 nm UV light for B. subtilis

Cobalt bipyridine is released as a byproduct of photolyzed caged oxygen. To ensure that oxygen and not the byproduct is the attractant, we performed an agarose-inplug bridge time-lapse dark-field microscopy assay [20]. LB growth medium and 10 mM serine were used as positive controls, with buffer as a negative control. UV light is the third possible stimulus, which might cause a change in tumble frequency. To rule out this possibility, we performed a flash experiment with buffer replacing caged oxygen.

2.5. Error calculations

The mean rcd value (rcdmean) and rcd standard deviation value (rcdSD) was calculated using rcd values between the 2–5 s baselines. A truncated rcd plot between 5–7.5 s range with a cubic polynomial algorithm to generate a best-fit curve yielded the peak rcd value (rcdpeak). The error in rcd = rcdpeak−rcdmean ± rcdSD. The standard deviations produced with the best-fit curve were small (< 0.005 s), and were therefore ignored in these calculations. We found a 0.02-s delay in shutter signal speed and corrected the results accordingly. Excitation response rates, kex, were determined according to Khan et al.

![Graph](image-url)
al. and MacArthur et al. [14,15] from single exponential fits. The response half time, $t_{1/2} = 1/2(k_{ex})$, was determined by a logistic fit according to Renate et al. [21].

3. Results and discussion

Baracchini and Sherris were among the first to describe the aerophilic nature of Bacteria [2]. In this report we demonstrate the measurement of excitation responses to discrete stimuli produced by photolysis of caged oxygen in Bacteria using computerized video analysis.

3.1. Oxygen sensing in B. subtilis

HemAT-Bs in B. subtilis binds oxygen reversibly and triggers aerotactic responses [5]. Thus, by using the caged oxygen compound to release diatomic oxygen at a given moment, it is possible to test behavioral responses directly related to oxygen-binding characteristics of HemAT-Bs. We have tested this method with wild-type B. subtilis and observed a rapid smooth swimming response towards photoreleased oxygen. Wild-type B. subtilis cells increase tumble frequency when flashed with 900 ms UV (360 ± 20 nm) in the absence of caged oxygen (data not shown). This result is consistent with previously observed swimming behavior of UV-irradiated bacteria [22–24]. When flashed with UV in the presence of caged oxygen, the cells exhibited a strong smooth response with an excitation time of 0.28 ± 0.04 s (Fig. 1A). A strain lacking in all ten transducers, Δten, did not respond to released oxygen (Fig. 1B), indicating that the presence of oxygen-sensing transducers is essential for aerotactic responses. Indeed, in a strain with overexpression of hemAT-Bs (Δten+hemAT-Bs), pho-
torelease of oxygen triggered a strong smooth response with an excitation time of $0.31 \pm 0.04$ s similar to that of the wild-type (Fig. 1C).

To further confirm that these responses are triggered by HemAT-Bs, we tested B. subtilis strain $\Delta ten^{+}$hemAT-Bs$_{H123A}$. We have previously demonstrated that His123 is a proximal residue in HemAT-Bs and plays an important role in oxygen binding [7]. Replacing this proximal histidine with an alanine residue destroys the heme-binding capability of HemAT-Bs [7]. Fig. 1D shows that the mutant strain $\Delta ten^{+}$hemAT-Bs$_{H123A}$ does not undergo an aerotactic response.

### 3.2. Oxygen sensing in E. coli and B. halodurans

To test our method in other bacteria, we analyzed aerotactic responses in E. coli and B. halodurans cells towards flash photoreleased oxygen. Wild-type E. coli RP437 showed a positive response to photoreleased oxygen with an excitation time of $0.21 \pm 0.04$ s (Fig. 2A). A single deletion of $tsr$ or $aer$ genes did not eliminate the aerotactic response (Fig. 2C,D). A strain lacking Tsr, RP5882 $\Delta (tsr)$, shows an excitation time of $0.22 \pm 0.04$ s (Fig. 2C), and BT3391 pTrc $\Delta (aer)$ shows an excitation time of $0.20 \pm 0.04$ s (Fig. 2D). Both the double mutant BT3312 $\Delta (aer tsr)$ (Fig. 2E) and the strain missing all five transducers BT3388 $\Delta (aer tar tsr trg tap)$, Fig. 2B) did not show significant response to photoreleased oxygen, indicating that oxygen-sensing transducers are required for aerotactic responses. Indeed, overexpression of $aer$ in BT3388 strain yielded a large decrease in red with an excitation time of $0.59 \pm 0.04$ s, confirming the oxygen-sensing capability of this receptor (Fig. 2F). Flash experiments without caged oxygen showed the expected negative response, indicating that the UV acted as a repellent (data not shown), as has been previously reported [22–24].

Our results showed that strains with Tsr adapted more slowly to changing oxygen concentrations than strains with Aer. Tsr-containing strains not only took longer to reach the pre-stimulus stage but also showed shorter excitation response times than did Aer-containing strains. When the transducer genes hemAT-Bs and aer were over-expressed, excitation times became longer ($\Delta ten^{+}$hemAT-Bs $\sim 0.31 \pm 0.04$ s and aer$^{+}$ $0.59 \pm 0.04$ s). Spudich and Koshland [25] showed that the duration of the smooth swimming response is proportional to receptor occupancy, i.e. the number of receptors titrated. The prolonged duration of the responses in these two strains (Fig. 2C,F) might be due to a larger number of Aer receptors that require a longer time for the adjustment to occur.

![Fig. 3. Response of B. halodurans C-125 to photoreleased oxygen. B. halodurans C-125 showed a decrease in rcd following a 100-ms flash in the presence of caged oxygen (A), but did not significantly respond when caged oxygen was absent (B).](image-url)
B. halodurans were able to respond to diatomic oxygen produced by photolysis of a caged oxygen compound. Therefore, the technique is applicable to a wide range of microorganisms.

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