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Role of Methylation in Aerotaxis in *Bacillus subtilis*

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Taxis to oxygen (aerotaxis) in *Bacillus subtilis* was characterized in a capillary assay and in a temporal assay in which the concentration of oxygen in a flow chamber was changed abruptly. A strong aerophilic response was present, but there was no aerophobic response to high concentrations of oxygen. Adaptation to a step increase in oxygen concentration was impaired when *B. subtilis* cells were depleted of methionine to prevent methylation of the methyl-accepting chemotaxis proteins. There was a transient increase in methanol release when wild-type *B. subtilis*, but not a *cheR* mutant that was deficient in methyltransferase activity, was stimulated by a step increase or a step decrease in oxygen concentration. The methanol released was quantitatively correlated with demethylation of methyl-accepting chemotaxis proteins. This indicated that methylation is involved in aerotaxis in *B. subtilis* in contrast to aerotaxis in *Escherichia coli* and *Salmonella typhimurium*, which is methylation independent.

Aerotaxis, the migratory response towards or away from oxygen, is a universal property of motile bacteria that enables the bacteria to move to a concentration of dissolved oxygen that is optimal for their preferred metabolism (4, 42). The attraction to oxygen requires the electron transport system and is coincident with changes in the proton motive force (18, 19, 36).

In a homogeneous environment, *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus subtilis* exhibit random walk motility: periods of smooth swimming interrupted periodically by tumbles, or chaotic movements that reorient the cell (6). Favorable stimuli, such as increasing attractant concentrations or decreasing repellent concentrations, reduce the probability of tumbling, thereby extending bacterial travel in the favorable direction. In a temporal assay, these bacteria respond to a step increase in attractant concentration by suppressing tumbling and swimming smoothly and to a step decrease in attractant concentration by continuous tumbling (23, 46) (for a review of chemotaxis, see references 7, 22, 24, and 43).

Both *E. coli* and *S. typhimurium* adapt to an increased concentration of attractant by restoring random swimming behavior. Adaptation to attractant occurs when the CheR protein, a chemotaxis-specific methyltransferase (39), transfers a methyl group from *S*-adenosylmethionine (17) to the γ-carboxyl group of glutamyl residues on the transmembrane chemotaxis receptor (MCP) (38, 48). Adaptation to an increased concentration of repellent occurs when the CheB protein, a methylesterase, hydrolyzes the glutamyl methyl esters to liberate methanol (41). The production of methanol can be followed by a perfusion assay that measures the time course of methanol release from bacteria during chemotaxis (14). An attractant added to *E. coli* cells inhibits methanol release during adaptation to the attractant, and removal of the attractant enhances methanol release during adaptation to the unfavorable (repellent) stimulus.

In contrast to methylation-dependent chemotaxis in which adaptation is dependent on receptor methylation, aerotaxis and chemotaxis towards substrates of the phosphoenolpyruvate:glycose phosphotransferase system in *E. coli* and *S. typhimurium* do not require methylation of a transmembrane receptor for adaptation (28). The means by which methylation-independent adaptation occurs in response to these chemostimulants remains unknown (2).

Sensory transduction in chemotaxis in *B. subtilis* requires methylation, but the role of methylation is clearly different from the role of methylation in *E. coli* and *S. typhimurium* (8). For example, when *B. subtilis* cells are stimulated with a chemoeffector during a perfusion assay, they release methanol after an attractant is added and after it is removed (45). Furthermore, taxis to glucose, a phosphotransferase substrate, is associated with methanol release in *B. subtilis* cells in contrast to *E. coli* cells (44). The distinctive pattern of methanol release in *B. subtilis* chemotaxis is also observed in chemotaxis in *Halobacterium salinarium*, suggesting that the sensory transduction mechanisms in the two species may be similar (1). A recent study in this laboratory showed that, in *H. salinarium*, aerotaxis involves methylation unlike aerotaxis in *E. coli* (20, 21). We investigated whether methylation was also involved in aerotaxis in *B. subtilis*.

Aerotaxis in *B. subtilis* was reported in several earlier studies. Baracchini and Sherris (4) observed banding in capillary tubes in aerotaxis assays of *B. subtilis*. Smooth swimming behavior was observed when anaerobic *B. subtilis* cells were aerated in a temporal assay of aerotaxis (25). In this study we characterize the aerotactic behavior and demonstrate that methylation is involved in aerotaxis in *B. subtilis*.

(A preliminary report of this work was presented at the 92nd General Meeting of the American Society for Microbiology [52].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. subtilis* OI1085 (*trpF7 hisH2 metC*) (47), *E. coli* RP437 (F⁻ *thi thr leu his met eda rpsL*) (32), and *S. typhimurium* ST23 (*hisF8786 thyA1981*) (3) are strains that are wild type for chemotaxis. *B. subtilis* OI1100 (OI1085 *cheR*) (47) is a chemotactic methyltransferase mutant. *B. subtilis* and *E. coli* strains were grown in Luria-Bertani broth supplemented with thiamine (0.5 μg/ml), and *S. typhimurium* was grown in nutrient broth supplemented with thymine (25 μg/ml). All bacteria were grown aerobically at 35°C. Spores of *B. subtilis* were produced in sporulation media (27) and diluted with sterile 100% glycerol in a 7:3 cell/glycerol ratio before freezing. Motile *B. subtilis* cells were obtained after overnight passage of frozen spores in Luria-Bertani broth and twice in mineral salts medium (30).

Behavioral assays. Temporal and spatial assays for aerotaxis were performed

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in a microchamber ventilated with prehumidified N_2 or O_2 gases as described previously (19, 35). Bacteria were observed with a Leitz Dialux dark-field video microscope. For behavioral assays, *B. subtilis* cells were grown in mineral salts medium, harvested in early logarithmic phase (optical density at 600 nm [OD_{600}], 0.3 to 0.4), and diluted approximately 33 fold (40 to 50 cells visible on the video monitor) for microscopic observation. For the temporal assay of a chemotaxis gradient, 1 μl of attractant or repellent was rapidly mixed with 9 μl of cell culture on a microscope slide. The duration of the behavioral response was defined as the interval between the addition of the chemoeffector or oxygen and restoration of tumbling in 50% of the cells. The tumbling frequency and speed of the bacteria were measured by the photographic procedure of Spudich and Koshland (35, 40) and by manual frame-by-frame analysis of video recordings (53). Spatial assays were also performed by placing cells (OD_{600}, 0.6; 2.5×10^8 cells per ml) in flat capillaries (inner dimensions, 0.2 by 2 by 50 mm; Vitro Dynamics Inc., Rockaway, N.J.) which were inserted into the microchamber ventilated with N_2, air, or O_2.

Methionine starvation. Cells were grown in mineral salts medium supplemented with 300 μM L-methionine and other auxotrophic requirements and harvested in exponential growth phase (OD_{600}, 0.5 to 0.6). The cells were washed by filtration (Millipore HA filter; pore size, 0.45 μm) and resuspended in mineral salts medium lacking L-methionine to a final OD_{600} of 0.1. Three to five milliliters of starved or unstarved cells was agitated at 35°C in 250-ml baffled Erlenmeyer flasks. Starvation was arbitrarily timed from the first wash in starvation medium.

Flow assay of [^{3}H]methanol evolution. The flow assay for methylesterase activity was essentially the procedure described by Kehry et al. (14) as modified by Lindbeck and coworkers (20, 21) for *H. salinarium* aerotaxis. The apparatus was further modified for this investigation. The tubing carrying perfusion buffer was replaced with 20-gauge stainless steel needle stock (Hamilton Co., Reno, Nev.), and buffer flow (1 ml/min) was regulated by negative pressure from a peristaltic pump placed after the filter in the flow apparatus. A four-way valve (model HVD4-5; Hamilton Co.), with low oxygen permeability, was used, and joints were coated with acrylic (nail polish) or Silly Putty to exclude oxygen. (A diagram of the modified apparatus may be obtained from the corresponding author.) Chemotaxis buffer (10 mM potassium phosphate [pH 7.0], 1 mM $MgSO_4$, 1 mM $(NH_4)_2SO_4$, 0.1 mM potassium-EDTA, 10 mM sodium lactate, 0.05% [vol/vol] glycerol, 0.14 mM $CaCl_4$) was sparged with N_2 or air (800 ml/min) for at least 5 h prior to the flow assay. The cells were exposed to an aerotactic stimulus (O_2) by switching the perfusion buffer from N_2-equilibrated to air-equilibrated chemotaxis buffer without stopping the pump.

B. subtilis cells growing in mineral salts medium ($OD_{600} \simeq 0.5$) were harvested, washed by filtration in cold chemotaxis buffer, and resuspended at an OD_{600} of 1.0 in chemotaxis buffer with chloramphenicol (0.2 mg/ml). After incubation for 10 min at 35°C in a shaking water bath, 85 μl of L-[*methyl*-^{3}H]methionine (70 to 85 Ci/mmol, 1 μCi/μl; DuPont NEN Research Products, Wilmington, Del.) was added, and incubation was continued for 20 min. Cells ($\sim 2 \times 10^9$) were loaded onto a prewetted Sartorius Minisart NML filter (diameter, 25 mm; pore size, 0.2 μm; Brinkmann Instruments, Inc., Westbury, N.Y.) and perfused with N_2-equilibrated chemotaxis buffer for 20 min to remove unincorporated [^{3}H]methionine. From each 500-μl fraction, collected in a 1.5-ml microcentrifuge tube, 350 μl was transferred to an open microcentrifuge tube that was immediately placed in a 20-ml plastic scintillation vial containing 7 ml of Universol (ICN Biomedicals, Costa Mesa, Calif.) scintillation cocktail. The scintillation vial was capped, placed in the dark at room temperature for 48 h, and counted for 5 min in a Beckman LS3801 liquid scintillation counter. Flow assays for *E. coli* and *S. typhimurium* cells were identical except that they were grown in Vogel-Bonner citrate medium E (49) supplemented with 1% glycerol, and auxotrophic requirements.

Oxygen measurements. Oxygen concentrations in the flow assay apparatus were measured in a flow cell machined from a 0.75 by 1.125 by 3 in. (ca. 1.9 by 2.9 by 7.6 cm) polycarbonate block into which a 20-gauge combination needle oxygen electrode (Diamond General Corp., Ann Arbor, Mich.) was inserted and connected to a Transidyne Chemical Microsensor (Transidyne General Corp., Ann Arbor, Mich.). Respiration was measured by using a Clark-type electrode as described by Shioi et al. (37).

In vivo methylation of methyl-accepting chemotaxis proteins. In vivo methylation was performed as described by Thoelke et al. (45), with the following changes. Cells were grown with vigorous agitation in baffled flasks containing 5 ml of Luria-Bertani broth, harvested at an OD_{600} of 0.2, washed by filtration, and resuspended at an OD_{600} of 1.0 in chemotaxis buffer containing chloramphenicol (0.2 mg/ml). After a 10-min incubation at 35°C, L-[*methyl*-^{3}H]methionine (20 μCi/ml) was added. Eight minutes later, excess nonradioactive methionine (10 μM) was added, and incubation was continued for another 2 min. A continuous stream of humidified N_2 or O_2 gas flowed over the vigorously stirred culture. Aliquots of cells were removed during the experiment and frozen rapidly. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (34), alkali-labile methyl groups were quantitated by the method of Chelsky et al. (9).

RESULTS

Aerotaxis assays. The respiration rate of *B. subtilis* was dependent on the growth phase, increasing from 0.47×10^{-10} μmol of O_2 per min per cell immediately after inoculation to a maximum of 1.85×10^{-10} μmol of O_2 per min per cell (1.86×10^6 molecules of O_2 per s per cell) in mid-exponential phase ($OD_{600} \sim 0.4$) (data not shown). Aerotaxis studies were performed on cells that were growing exponentially (OD_{600}, 0.3 to 0.6). Spatial aerotaxis assays were performed on a microscope stage using a microchamber that was ventilated with prehumidified nitrogen gas or air (see Materials and Methods). After a drop of the *B. subtilis* cell suspension in the N_2-ventilated microchamber became anaerobic, an air-filled capillary was inserted into the cell suspension. Within 1 min, wild-type *B. subtilis* cells formed a dense cluster around the mouth of the air-filled capillary that persisted for 2 h (data not shown). Cells in the cluster exhibited random swimming behavior within 0.5 mm of the air-filled capillary and random to tumbling behavior at the periphery of the cluster (0.5 to 0.8 mm) and tumbled frequently in a "clear" region beyond the cluster. Cells were nonmotile distally to the clear region. Cluster formation was reversible by switching the ventilation gas in the microchamber from N_2 to air and back to N_2 again, confirming that the cluster formed in response to a diffusion gradient of oxygen. In a second spatial assay, a flat capillary was filled with a cell suspension. The suspended cells consumed the oxygen in the interior of the capillary creating an oxygen gradient at the air-liquid interface. Cells formed a band at the gas-liquid interface (Fig. 1A). Cells swimming away from the aerotactic band into the clear region tumbled immediately. Cells unable to return to the aerotactic band after swimming past the clear region (approximately 3 mm from the interface) became nonmotile and were trapped by the anaerobic conditions. At similar cell densities, a *cheR* mutant (OI100) showed less accumulation at the mouth of an air-filled capillary (data not shown) or at the air-liquid interface (Fig. 1B), although swimming behavior in the cluster was normal.

In a temporal assay, a thin film of bacterial suspension was placed in the microchamber. The chamber was ventilated with N_2 until the cells became anaerobic, and then air or 100% O_2 was introduced. The response of the bacteria was observed through the microscope and video recorded. Wild-type *B. subtilis* cells tumbled constantly in response to N_2. The cells partially adapted to anaerobiosis after approximately 1 min (Fig. 2); however, tumbling frequency remained very high until the cells became nonmotile after approximately 10 min. The cells showed a smooth swimming response to 21% oxygen. The adaptation time to addition of oxygen in wild-type cells varied from 25 to 35 s depending on the experimental conditions and the method used to determine the tumbling frequency (Fig. 2 and 3). There was a 4-s delay in a smooth swimming response after substitution of air for N_2. Anaerobically paralyzed cells showed a twofold increase in the delay time over cells ventilated with N_2 for 2 min (the normal interval of N_2 ventilation). The swimming speed of 38 ± 5 μm/s in unstimulated cells decreased after 1 min to 21 ± 5 μm/s in cells adapted to anaerobiosis and increased to 40 ± 6 μm/s in smooth swimming cells that were responding to exposure to air. Similar changes in the swimming speed were observed for both wild-type and *cheR* cells. The *cheR* mutant which is deficient in methylation exhibited a shorter response (17 to 23 s) response to 21% oxygen and a delay (9 to 10 s) before the smooth swimming response was observed. Adaptation to withdrawal of oxygen in the *cheR* mutant appeared to be faster and more pronounced than in the wild-type strain (Fig. 2). These findings are consistent with the results for the spatial assays in which an impaired aerotaxis behavior results from a *cheR* mutation in the methylation pathway (Fig. 1).

High concentrations of O_2 (100% O_2, 1.2 mM dissolved

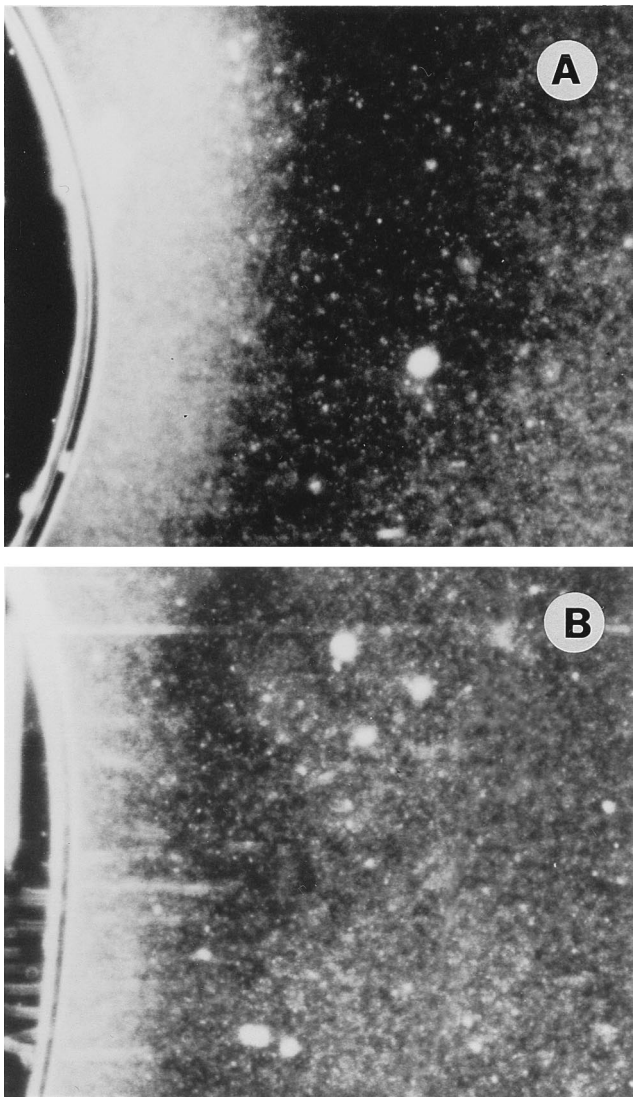

FIG. 1. Spatial assays of aerotaxis. *B. subtilis* wild-type (OI1085) (A) or *B. subtilis cheR* (OI1100) (B) cells were loaded into a flat capillary tube and photographed after 1 min. The air-liquid interface is visible on the left. See Materials and Methods for a description of the experimental conditions.

oxygen), which cause an aerophobic response in *E. coli*, did not act as a repellent for *B. subtilis* in either spatial or temporal assays, confirming an earlier report (35). Instead, a smooth swimming (attractant) response similar to the response to air was observed when the ventilating gas in the temporal assay was switched from N_2 to O_2. No statistically significant change in swimming behavior was noted when the ventilating gas was switched from O_2 to air or from air to O_2; the cells tumbled immediately after a switch from O_2 to N_2.

Chemotaxis in *cheR* mutants. *B. subtilis* OI1085 wild-type cells showed a 36 ± 2 s smooth swimming chemotaxis response to 32 μM asparagine in agreement with a previous report (29). *B. subtilis cheR* mutants exhibit random to tumbling swimming behavior (16) and swarmed in semisoft agar to approximately 70% of the diameter of wild-type swarms after 8 h. This is in contrast to *E. coli* and *S. typhimurium cheR* mutants which do not tumble and do not migrate through semisoft agar (50). As in the response to oxygen, the response of *cheR* mutants to 32 μM asparagine (12 ± 1 s) was shorter than that of wild type cells (38 ± 6 s) (47).

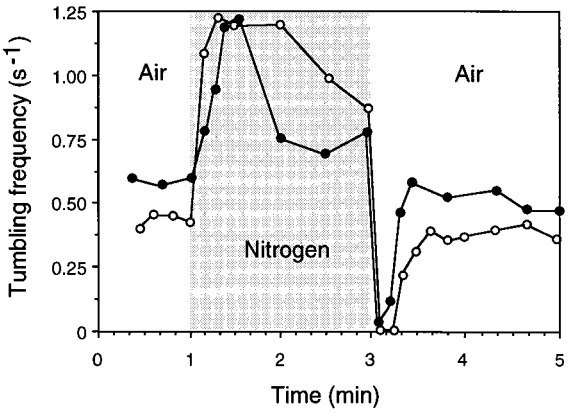

FIG. 2. Temporal assay of aerotaxis. Ten microliters of a wild type *B. subtilis* OI1085 (○) or *cheR* mutant (●) cell suspension was placed in a microchamber and equilibrated with air for 1 min or more before observation commenced. After the motility of the bacteria in air was observed for an additional minute, the chamber was ventilated with N_2 gas (shaded area) for 2 min, and then air was allowed to reenter the chamber. The tumbling frequency of the cells was calculated by frame-by-frame analysis as described in Materials and Methods.

Effects of methionine starvation on *B. subtilis* behavior. Cells were depleted of methionine as described in Materials and Methods. After 2 h of methionine starvation, *B. subtilis* OI1085

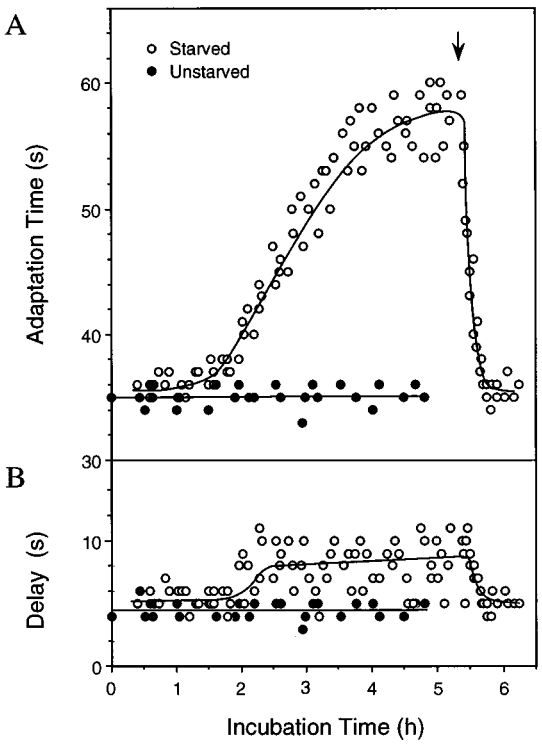

FIG. 3. Effect of methionine starvation on aerotaxis. (A) The time interval required for *B. subtilis* OI1085 (*metC*) cells to adapt to a step increase in oxygen concentration (N_2 to air) was determined as a function of the time of incubation in methionine-deficient media (○) or in methionine-containing media (●). The data are from three experiments performed on different days. Methionine (300 μM) was added at 5.33 h (arrow). (B) Delay before smooth swimming was detected in the temporal aerotaxis assay of starved (○) and unstarved (●) cells. The adaptation time was determined by the photographic procedure (35, 40).

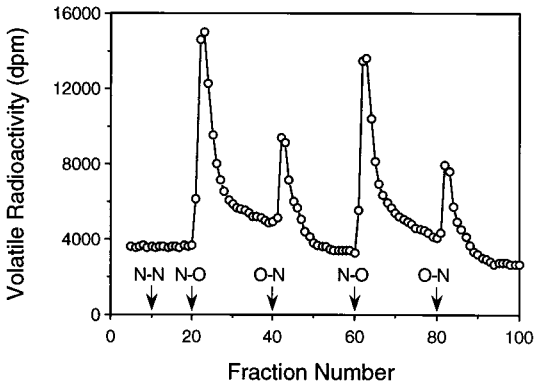

FIG. 4. Effect of oxygen stimulation on [^{3}H]methanol production in *B. subtilis* cells. [^{3}H]methionine-labeled *B. subtilis* OI1085 cells (2×10^9) were loaded onto the filter in the flow assay apparatus and perfused. N-N, N-O, and O-N, switch from one N_2-equilibrated buffer line to another, from N_2- to air-equilibrated buffer, or from air- to N_2-equilibrated buffer, respectively. See Materials and Methods for a description of the experimental protocol. Volatile radioactivity was determined for each fraction.

(*metC*) cells demonstrated a prolonged smooth swimming response in a temporal aerotaxis assay. Adaptation times increased as methionine starvation progressed but did not change in the unstarved control cells (Fig. 3A). Although no direct measurements of intracellular methionine or *S*-adenosylmethionine concentrations were made, the absence of cell growth in the starved culture indicated that methionine was depleted. Normal growth and adaptation times were restored after 300 μM methionine was added to the methionine-starved cells. A concurrent change was observed in the delay before the aerotactic response was detected (Fig. 3B). The delay was more variable in the methionine-starved cells than in the control cells. The addition of methionine to the methionine-starved cells returned the delay to prestarvation intervals and decreased the variation. Methionine starvation also increased the adaptation time to 32 μM asparagine (data not shown), confirming an earlier report (29). The effect was more pronounced for asparagine than in aerotaxis.

Flow assay of methanol evolution. Lindbeck (working in this laboratory) first modified the flow assay of Kehry et al. (14) for investigation of aerotaxis in *H. salinarium* and was able to demonstrate the release of small peaks of volatile radioactivity after oxygen stimulation (20). The apparatus was further modified for this investigation as described in Materials and Methods. *B. subtilis* cells were preincubated with L-[*methyl*-^{3}H]methionine, loaded onto a membrane filter, and perfused with anoxic buffer followed by air-equilibrated buffer. The release from the cells of volatile radioactivity into the perfusate was monitored. Oxygen measurements indicated that 100% air saturation was attained in less than 30 s, or the duration of one flow assay fraction, after the switch from N_2- to air-equilibrated buffer. However, to ensure that the cells were completely anaerobic after the switch from air- to N_2-equilibrated buffer, the cells were perfused with N_2-equilibrated buffer for at least 5 min (10 fractions).

Oxygen stimulation of *B. subtilis* cells caused transient changes in [^{3}H]methanol evolution. Methanol peaks were observed after the addition and after withdrawal of air-equilibrated buffer (Fig. 4). Peaks were larger after the addition than after the removal of air-equilibrated buffer, regardless of the order of these stimuli. Identification of the volatile radioactive fraction as methanol was established by derivatizing the flow assay fractions with 3,5-dinitrobenzoylchloride and separating

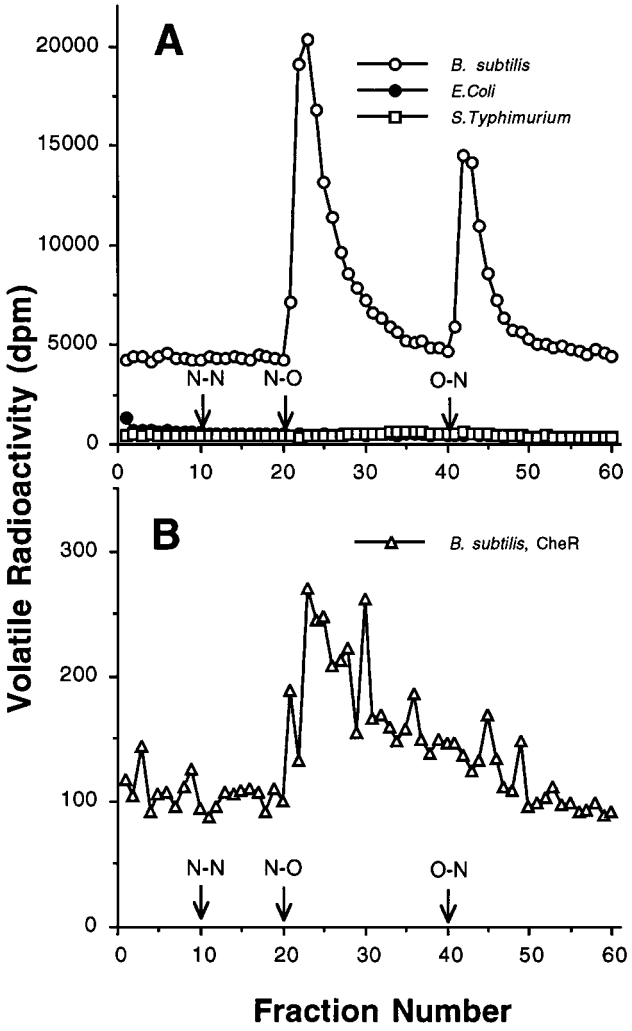

FIG. 5. Comparison of aerotaxis flow assays for various bacterial strains. Flow assays of methanol evolution in response to changes in oxygen concentration were performed as described in Materials and Methods. (A) *B. subtilis* OI1085, *E. coli* RP437, and *S. typhimurium* ST23, strains wild type for chemotaxis. (B) *cheR* mutant *B. subtilis* OI1100. Note the 33× difference in ordinate scales. N-N, N-O, and O-N, switch from one N_2-equilibrated buffer line to another, from N_2- to air-equilibrated buffer, or from air- to N_2-equilibrated buffer, respectively.

the derivatives by thin-layer chromatography (26, 33, 51). No derivative of methanethiol was detected. In a control experiment, the switch from one N_2-equilibrated buffer line to another did not cause any transient methanol production (Fig. 4). If oxygen is slowly diffusing into the N_2 line or switching valve, the cells will show an oxygen response when perfusion is switched from an active N_2-equilibrated line to one that has been static for 20 min or longer (see also reference 21). The volatile radioactivity released was highest in cells that were labeled with [^{3}H]methionine for 20 min. As a result, cells for flow assays were routinely labeled for 20 min. Essentially no methanol evolution was observed in a methyltransferase mutant (Fig. 5). The mutant is leaky (approximately 10% of wild-type methyltransferase activity) (12) and did show a small peak on addition of air-equilibrated buffer (Fig. 5B). Identical experiments were carried out with wild-type *E. coli* (RP437) and *S. typhimurium* (ST23) cells, in which no methanol peaks were observed in response to changes in the oxygen concentration

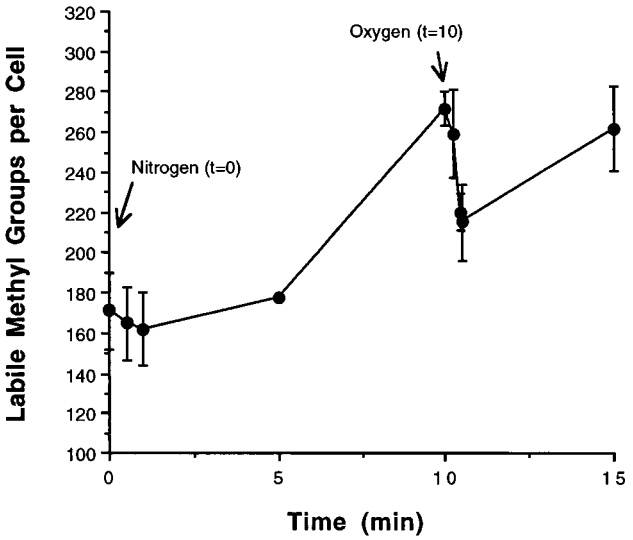

FIG. 6. Changes in total MCP methylation during aerotaxis. Wild-type cells (*B. subtilis* OI1085) were labeled with [^{3}H]methionine as described in Materials and Methods. The cell suspension was made anaerobic by flowing N_2 over the vigorously stirred cells. After 10 min (t = 10) the cells were aerated by O_2 for 5 min. Aliquots of cells removed at the times shown were analyzed by SDS-PAGE, and the alkali-labile methyl groups were quantitated.

(Fig. 5A). These results, along with the previous findings that *cheR*, *cheB*, and MCP-deficient mutants demonstrate normal aerotaxis (10, 28), support the hypothesis that methylation is not required for aerotaxis in *E. coli* and *S. typhimurium*.

In addition to aerotactic stimulation in flow assays, [^{3}H]methionine-labeled *B. subtilis* cells were perfused with the phosphotransferase sugar mannitol (0.64 mM; 20-fold excess over the concentration required to saturate the response [31]). Wild-type but not *cheR* cells produced volatile peaks (not shown). Thoelke et al. (44) previously demonstrated that chemotaxis to glucose, which is a phosphotransferase substrate in *B. subtilis*, is also methylation dependent. That finding together with the present results with mannitol indicates that phosphotransferase chemotaxis in *B. subtilis* is methylation dependent.

Changes in methylation with oxygen stimulation. As in *E. coli* and *S. typhimurium*, there are *B. subtilis* proteins that can be labeled in vivo with [^{3}H]methionine in the absence of protein synthesis or labeled in vitro with *S*-adenosyl[*methyl*-^{3}H]methionine (47). In this study, three distinct bands with apparent molecular weights of 69,000, 71,000, and 73,000 were observed in the chemotactic wild-type *B. subtilis* (OI1085) cells but not in the *cheR* (OI1100) mutant (data not shown). To compare the methanol produced in aerotaxis flow assays with in vivo MCP demethylation, [^{3}H]methionine-labeled cells were equilibrated with N_2 and then aerated. Aliquots were removed at intervals during the incubation, rapidly frozen (<2 s in a dry ice-ethanol bath), and analyzed by SDS-PAGE. The alkali-labile methyl groups in the gels were quantitated (9). The replacement of N_2 gas with 100% O_2 gas resulted in a rapid increase in oxygen concentration; an oxygen concentration similar to that in air-equilibrated buffer was achieved after 15 s.

The total of alkali-labile methyl groups was changed by switching the ventilating gas from nitrogen to oxygen (Fig. 6). After 1 min of nitrogen stimulation, between 7 and 10 methyl groups per cell were lost. This was not statistically significant for the number of determinations that were made. Within 30 s after oxygen stimulation, 50 to 57 methyl groups per cell were lost ($P < 0.05$). After the cells adapted to the aerotactic stimulus (typically 35 to 40 s), there was a recovery of methylation. The changes in MCP methylation were compared to the total methanol produced in the flow assay. Integrated values from peaks of methanol released during the flow assays represented losses of 11 to 13 molecules of methanol per cell and 65 to 71 molecules of methanol per cell after switches to N_2- and air-equilibrated buffers, respectively. Allowing for losses during sample preparation and gel electrophoresis, the MCP demethylation and the flow assay values appear to be in agreement.

DISCUSSION

B. subtilis cells, as do *E. coli* and *S. typhimurium*, respond to a temporal increase in oxygen concentration by swimming smoothly and to a decrease in oxygen by tumbling (Fig. 2). These bacteria rapidly form aerotactic bands in spatial gradients of dissolved oxygen (Fig. 1A). Cells that swim away from the aerotactic band tumble immediately and continue tumbling until they can reorient their swimming direction to return to the aerotactic band. Thus, like other aerotactic bacteria, *B. subtilis* can migrate to the region where the oxygen concentration is optimal for metabolism.

Several features differentiate aerotaxis in *B. subtilis* from the aerotaxis behavior of enteric bacteria. First, in the aerotactic temporal response to an oxygen gradient in *B. subtilis* the adaptation time is longer than that of *E. coli* and *S. typhimurium*: the latter respond for 10 to 15 s (19), whereas *B. subtilis* responded for 25 to 35 s when anaerobic cells were exposed to oxygen. Secondly, in a spatial gradient of dissolved oxygen, *B. subtilis*, an obligate aerobe, congregated at the air-liquid interface (Fig. 1A) unlike the facultatively anaerobic *E. coli* and *S. typhimurium*, which formed a band at 50 μm from the interface (35), or the microaerophilic *Azospirillum brasilense*, which formed a band at 200 to 1,000 μm from the interface (5, 13). The position of the aerotactic band reflects the oxygen requirement for optimal growth of the bacteria. Thirdly, neither aerobic nor anaerobic *B. subtilis* cells were repelled by high oxygen concentrations, confirming an earlier report (35). Instead, a smooth swimming response occurs when the gas ventilating the *B. subtilis* cells in a temporal assay changes from nitrogen to 100% oxygen. This implies that the toxic by-products of oxygen metabolism (superoxide anion, hydroxyl radical, or hydrogen peroxide [11]) generated during respiration are apparently not sufficiently deleterious to have elicited an avoidance response in *B. subtilis*.

The behavior of *B. subtilis* in anaerobiosis is also different from that of *S. typhimurium*, *E. coli*, and *Bacillus cereus*. When ventilated with N_2 gas, *B. subtilis* cells showed a very high tumbling frequency until they were paralyzed by prolonged anaerobic exposure. In anaerobic conditions, *B. cereus* cells become immotile and then adapt and resume random motility (18); *S. typhimurium* and *E. coli* briefly tumble, swim smoothly for 2 to 3 min, and return to random motility (15, 19).

This study presents evidence that methylation is required for normal adaptation of *B. subtilis* cells to an increase in the oxygen concentration, in contrast to aerotaxis in *E. coli* and *S. typhimurium*, which is methylation independent. Methionine starvation depletes intracellular *S*-adenosylmethionine, the substrate for the *cheR* methyltransferase, and is therefore a useful tool for studying the involvement of methylation in chemotaxis. Methionine starvation prolonged the smooth swimming aerotactic response of *B. subtilis* (Fig. 3). Normal response times were restored only after the addition of methionine to the starved cells, in agreement with a mechanism for adaptation to oxygen that requires methylation or demethylation. These changes are similar to those observed for methyl-

ation-dependent chemotaxis. In contrast, *S*-adenosylmethionine depletion in *S. typhimurium* does not affect aerotaxis (28). The impaired aerotactic response of a *B. subtilis* methyltransferase (*cheR*) mutant in spatial gradients of oxygen (Fig. 1) and in temporal assays also suggests that methylation of an MCP is required for normal aerotaxis. However, the responses of the *cheR* mutant to an abrupt increase in oxygen concentration were of shorter duration than in wild-type *B. subtilis* cells, whereas methionine starvation produced longer responses. The reason for this anomalous observation is not clear, but a similar result was observed in chemotaxis, with the *cheR* mutant *B. subtilis* OI1100 showing shorter responses to a temporal gradient of asparagine. The role of methylation in chemotaxis has not been fully elucidated in *B. subtilis*, but there are significant differences from the mechanism in *E. coli* (8). In the present study of aerotaxis we demonstrated similar methylation-related phenomena in aerotaxis and chemotaxis in *B. subtilis* but did not establish a mechanism for methylation in aerotaxis. There is not an absolute requirement of methylation for the excitation phase of the aerotactic response, but methylation appears to be associated with some aspect of normal adaptation.

The requirement for methylation in *B. subtilis* aerotaxis is further supported by transient increases in methanol evolution following oxygen addition and oxygen withdrawal in a flow assay (Fig. 4). The durations are comparable for peak methanol production and the smooth swimming aerotactic response. The methyltransferase protein (*cheR* gene product) is required for the air-stimulated methanol peaks in the aerotaxis flow assays (Fig. 5). Quantitation of changes in MCP methylation (Fig. 6) in aerotaxis accounted for the methanol evolved in flow assays performed under similar cell conditions. In contrast to methylation-dependent aerotaxis in *B. subtilis*, no methanol peaks were evident in similar flow assays of aerotaxis in *E. coli* and *S. typhimurium*. This extends a previous finding (28) that methylation is not involved in aerotaxis in these bacteria.

Collectively, the methionine starvation experiments, behavioral studies with a *cheR* mutant, flow assays, and the in vivo MCP methylation experiments support a role of methylation/demethylation in aerotaxis in *B. subtilis*. Lindbeck (20, 21) (working in this laboratory) previously demonstrated that aerotaxis in *H. salinarium* is also dependent on methylation. Although these are the only two known examples of bacteria in which aerotaxis is associated with methylation, it is likely that they represent a much larger class of bacteria. Taxis by *B. subtilis* to mannitol and glucose, phosphotransferase substrates, is also methylation dependent (44) unlike phosphotransferase chemotaxis in *E. coli* and *S. typhimurium* (28). Involvement of methylation in aerotaxis, phosphotransferase chemotaxis, and chemotaxis in *B. subtilis* suggests that these pathways converge at an early stage in the signal transduction pathway.

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