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Amino Acid Efflux in Response to Chemotactic and Osmotic Signals in *Bacillus subtilis*

LAURENCE S. WONG,† MARK S. JOHNSON,* LAWRENCE B. SANDBERG, AND BARRY L. TAYLOR

Department of Microbiology and Molecular Genetics, Loma Linda University, Loma Linda, California 92350

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We observed a large efflux of nonvolatile radioactivity from *Bacillus subtilis* in response to the addition of 31 mM butyrate or the withdrawal of 0.1 M aspartate in a flow assay. The major nonvolatile components effluxed were methionine, proline, histidine, and lysine. In studies of the release of volatile radioactivity in chemotaxis by *B. subtilis* cells that had been labeled with [^{3}H]methionine, the breakdown of methionine to methanethiol can contribute substantially to the volatile radioactivity in fractions following addition of 0.1 M aspartate. However, methanol was confirmed to be released after aspartate addition and, in lesser quantities, after aspartate withdrawal. Methanol and methanethiol were positively identified by derivitization with 3,5-dinitrobenzoylchloride. Amino acid efflux but not methanol release was observed in response to 0.1 M aspartate stimulation of a *cheR* mutant of *B. subtilis* that lacks the chemotaxis methylesterase. The amino acid efflux could be reproduced by withdrawal of 0.1 M NaCl, 0.2 M sucrose, or 0.2 M xylitol and is probably the result of changes in osmolarity. Chemotaxis to 10 mM alanine or 10 mM proline resulted in methanol release but not efflux of amino acids. In behavioral studies, *B. subtilis* tumbled for 16 to 18 s in response to a 200 mosM upshift and for 14 s after a 20 mosM downshift in osmolarity when the bacteria were in perfusion buffer (40 mosM). The pattern of methanol release was similar to that observed in chemotaxis. This is consistent with osmotaxis in *B. subtilis* away from an increase or decrease in the osmolarity of the incubation medium. The release of methanol suggests that osmotaxis is correlated with methylation of a methyl-accepting chemotaxis protein.

Escherichia coli and *Salmonella typhimurium* adapt to chemotactic stimuli by methylating or demethylating the chemotaxis receptor, also known as a methyl-accepting chemotaxis protein (for a review, see references 5 and 28). The level of methylation increases in cells adapting to an increase in attractant concentration or adapting to a decrease in repellent concentration. Demethylation of the receptor produces methanol during adaptation to an increase in repellent concentration or to a decrease in attractant concentrations (26). In *E. coli* and *S. typhimurium* exposed to an attractant in a flow assay, there is a transient decrease in methanol release (12). Subsequent withdrawal of the attractant or addition of a repellent results in a transient increase in methanol release.

Bacillus subtilis has a different and more complex pattern of methylation in chemotaxis (for a review, see reference 6). In the flow assay for chemotaxis, *B. subtilis* cells show a transient increase in methanol release after addition of aspartate and also after withdrawal of aspartate (30). A similar pattern of transient methanol increase after attractant addition and attractant withdrawal is found in *Halobacterium salinarium* (2). Recent studies in this laboratory have demonstrated a transient methanol release in *B. subtilis* and *H. salinarium* after a step increase and after a step decrease in oxygen concentration (17, 38). This and other evidence established that aerotaxis in *B. subtilis* and *H. salinarium* is methylation dependent, unlike aerotaxis in *E. coli* and *S. typhimurium*, which is methylation independent (19). The present study was undertaken to investigate the mechanism responsible for this anomalous pattern of methanol release in aerotaxis and chemotaxis in *B. subtilis*.

* Corresponding author. Phone: (909) 824-4480. Fax: (909) 824-4035. Electronic mail address: mJohnson@ccmail.llu.edu.

† Present address: Department of Medical Biochemistry, The University of Calgary, Calgary, Alberta T2N 4NI, Canada.

Aspartate is the strongest attractant for *B. subtilis*, but the dissociation constant for the response is 2.9×10^{-2} M, necessitating the use of high concentrations of aspartate for a maximum response (9). Butyrate is a strong repellent of *B. subtilis* but is also used at high concentrations (11). As a result, the colligative properties of aspartate and butyrate stimuli must be considered. Numerous responses in bacteria to osmotic changes have been reported. One of these is the efflux of amino acids. Passive efflux of amino acids in response to a hypoosmotic shift has been observed for proline, glutamate, lysine, and other amino acids in diverse bacteria (for a review, see reference 13). These amino acids, together with K^+, trehalose, and glycine betaine, are osmoprotectants that maintain turgor, and their concentration in the cell is adjusted as the osmotic strength of the external medium changes.

(A preliminary report of this work was presented at the 85th Annual Meeting of the American Society for Biochemistry and Molecular Biology [37].)

MATERIALS AND METHODS

Strains and growth conditions. *B. subtilis* OI1085 (*trpF7 hisH2 metC*) (34) and *E. coli* RP437 (F$^-$ *thi thr leu his met eda rpsL*) (20) are strains that are wild type for chemotaxis; *B. subtilis* OI1100 (OI1085 *cheR*) (34) is deficient in the methyltransferase for chemotaxis. The strains were grown at 35°C in LB broth supplemented with required nutrients as described previously (38).

Flow assay of [^{3}H]methanol evolution. The flow assay for CheB methylesterase activity (12) was modified to measure aerotaxis as described elsewhere (38). Vapor transfer of [^{3}H]methanol was measured after 48 h. The following scintillation cocktails were investigated for efficiency of methanol transfer: cocktails for aqueous and nonaqueous samples were Ecolume and Ecolite (ICN Biomedicals, Costa Mesa, Calif.), Ready Safe (Beckman, Fullerton, Calif.), and ScintiSafe 30% (Fisher Scientific Co., Pittsburgh, Pa.); cocktails for aqueous samples were AquaMix, Cytoscint ES, Betablend, and Universol ES (ICN Biomedicals) and ScintiSafe Gel (Fisher Scientific Co.); cocktails for nonaqueous samples were Betamax and Betamax ES (ICN Biomedicals) and Ready Organic (Beckman). Universal ES was adopted for use in this study. Various scintillation vials were tested for evaporation of [^{3}H]methanol and found to have a significant leakage

rate. High-density polyethylene vials (20 ml, narrow mouth; Fisher Scientific Co.) were selected for use in this study because they had a minimum loss of [^{3}H]methanol.

Identification of nonvolatile radioactivity. The nonvolatile peak in the chemotaxis flow assay was chromatographed on 3MMChr chromatography paper (Whatman) that was predeveloped with deionized H_2O and developed with *n*-butanol–acetic acid–H_2O (12:3:5) in a chromatography chamber for 3.5 h (25). Nonradioactive methionine, methionine sulfoxide, and homocysteine were included as standards. Nonradioactive standards were visualized with 0.25% ninhydrin. The radioactive lanes were cut into 1-cm sections, and radioactivity was counted in Universol ES cocktail after 24 h.

The nonvolatile peaks were further analyzed on a Biotronik 5001 amino acid analyzer (Biotronik, Munich, Germany) as described by Sandberg and Davidson (23). Twenty microliters of radioactive sample (~1 μCi) was mixed with 100 μl of known amino acid standards (2.5 μM each), and a 50-μl aliquot was injected onto a sulfonated polystyrene high-performance liquid chromatography (HPLC) column. Fractions were collected every 0.5 min, and 180 μl of each fraction was transferred directly into 10 ml of Universol ES cocktail and counted for 10 min after standing for 24 h at room temperature.

Identification of volatile radioactivity. The volatile compounds in the flow assay were derivatized with 3,5-dinitrobenzoyl chloride (DNB) by a modification of published procedures (18, 32). Peak and nonpeak fractions were pooled separately, divided into 0.5-ml aliquots, and placed in 1.5-ml microcentrifuge tubes. Each tube was vortexed with 0.5 ml of ice-cold 40% NaOH before 0.4 g of DNB (Sigma Chemical Co., St. Louis, Mo.) was added. The mixture was vortexed for 10 min. One half milliliter of ice-cold deionized H_2O was added to the tube, and the contents were vortexed and centrifuged. The supernatant was discarded, and the pellet was washed four times with 0.5 ml of ice-cold deionized H_2O. The pellet was then dried for 30 min in a SpeedVac vacuum centrifuge (Savant Instruments, Inc., Farmingdale, N.Y.) and resuspended in 0.5 ml of ethyl acetate by agitation on a rotary platform overnight. Resuspended pellets from the same pool were combined, lyophilized, and resuspended in 50 to 100 μl of ethyl acetate.

The derivatized samples (1 to 5 μl) were spotted on a Silicagel 60 thin-layer chromatography plate (20 by 20 cm; Eastman Kodak Co., Rutherford, N.J.). Nonradioactive CH_3O- and CH_3S-3,5-dinitrobenzoate derivatives were added as standards. Sodium methanethiolate (CH_3SNa; Fluka Chemical Corporation, Buchs, Switzerland) was dissolved in deionized water to release the highly volatile methanethiol before derivatization with DNB. The chromatogram was developed with 100% toluene for approximately 1.5 h in a thin-layer chromatography chamber (procedure modified from that in reference 22). The nonradioactive standards were visualized by UV light, and the R_f was calculated for each sample. The radioactive lanes were cut into 0.5- or 1-cm sections along the vertical axis, placed into 5 ml of Universol cocktail, and counted after 24 h.

Temporal assay for osmotaxis. The temporal assay used to measure the response of bacteria to an osmotic shift was similar to one described previously (16). Bacteria (5 to 9 μl) in chemotaxis buffer (~40 mosM) were mixed on a microscope slide with a concentrated solution of the solute to be tested for osmotic effects (final volume, 10 μl). The bacterial behavior was observed with a Leitz dark-field video microscope. The adaptation time during the chemotactic response was measured with a time-date generator (Panasonic model WJ-810) to superimpose the time on each frame of the videorecording.

RESULTS

Flow assay of [^{3}H]methanol evolution. The flow assay is widely used to measure the activity in vivo of the CheB methylesterase in chemotaxis (12). Bacteria that have been labeled with L-[*methyl*-^{3}H]methionine are loaded onto a membrane filter and perfused with buffer that allows the addition or withdrawal of a chemoeffector. The production of [^{3}H]methanol by the bacteria in response to a step increase in repellent concentration or a step decrease in attractant concentration is monitored by analysis of the perfusate. Aliquots of perfusate are transferred to an uncapped microcentrifuge tube (ship) that is placed upright in a 20-ml scintillation vial that contains scintillation fluid (ocean). Vapor transfer of [^{3}H]methanol from the ship to the ocean reaches equilibrium in 36 to 48 h at room temperature (7, 12; this study) (data not shown). The conditions for optimal transfer of methanol to the scintillation fluid were reexamined (see also reference 12). The endpoint for methanol transfer was dependent on the ocean-ship ratio (Fig. 1). At equilibrium, 75% of the methanol was transferred to the scintillant when the ocean-ship ratio was 80:1 and 90% was transferred at a ratio of 160:1. The methanol transferred approximated a first-order function at ocean-ship ratios below 40.

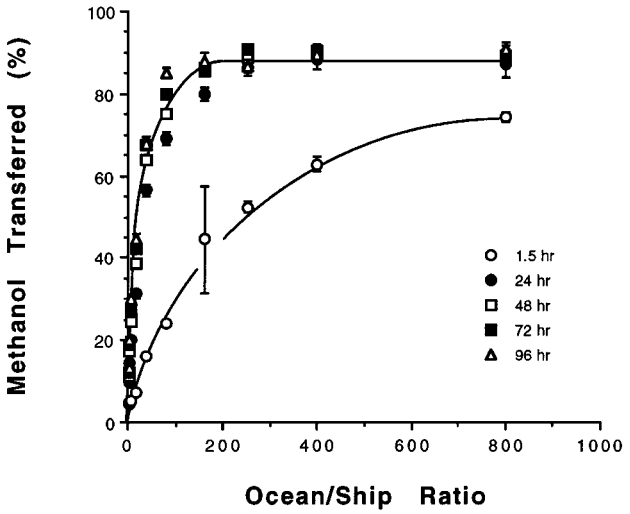

FIG. 1. Effect of ocean-ship ratio on transfer of [^{14}C]methanol at room temperature. Approximately 4,000 cpm of [^{14}C]methanol was dissolved in various volumes of chemotaxis buffer in microcentrifuge tubes (ship) and placed in 20-ml plastic scintillation vials, each containing a known volume of Universol scintillation cocktail (ocean). Each datum point was the average of three determinations. Error bars indicate the standard deviation of the mean. See Materials and Methods for details of the procedure.

A total of 12 commercial scintillation cocktails were investigated for efficiency of methanol transfer (Materials and Methods). The endpoint (65% transferred at a 60:1 ocean-ship ratio) was similar for nine cocktails that are formulated for aqueous samples (data not shown). The endpoint was 25 to 30% transferred in three cocktails that are designed for nonaqueous samples. Universol ES (ICN Biomedicals) was selected for this study because it has a large sample-loading capacity (40%, vol/vol) and is nontoxic and biodegradable.

Chemotactic responses to aspartate and butyrate. Flow assays of the chemotactic response of *B. subtilis* to 0.1 M aspartate (Fig. 2A) confirmed the paradoxical release of volatile radioactivity in response to both favorable and unfavorable stimuli, as reported previously by Thoelke et al. (30). Volatile radioactivity was observed after addition of 31 mM butyrate but not following butyrate withdrawal. In an investigation of this phenomenon, the total (volatile and nonvolatile) radioactivity in the fractions from the flow assay was determined. After vapor transfer and counting of volatile radioactivity, the scintillation vials were inverted, the contents of the ship and ocean were mixed, and the vials were recounted for total radioactivity (Fig. 2B). The total radioactivity was 100- to 200-fold greater than the volatile radioactivity. A large peak of nonvolatile radioactivity was released in response to an unfavorable stimulus such as withdrawal of the attractant aspartate or addition of the repellent butyrate (Fig. 2B). Repeated cycles of addition and withdrawal of aspartate resulted in progressively diminishing peaks of both volatile and nonvolatile radioactivity (Fig. 3).

Identification of nonvolatile radioactivity. The large peak of total radioactivity released in response to withdrawal of aspartate was further analyzed by heating fractions from the flow assay in a boiling-water bath to remove all volatile components. The amount of nonvolatile radioactivity that was released by *B. subtilis* OI1085 cells after aspartate withdrawal greatly exceeded the amount of volatile radioactivity released (Fig. 4A). By comparison, the radioactivity released by *E. coli* RP437 in response to serine (attractant) withdrawal or leucine (repellent) addition was entirely volatile (Fig. 4B and C).

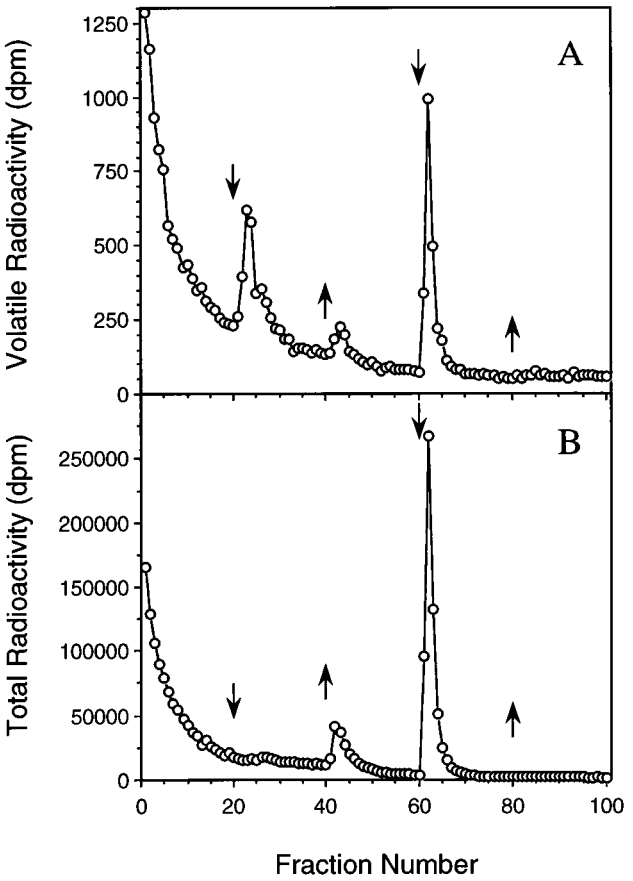

FIG. 2. Volatile and total radioactivity released by wild-type *B. subtilis* OI1085 in response to aspartate and butyrate addition and removal. Approximately 10^9 cells grown in Luria-Bertani broth at 35°C and harvested at an optical density at 600 nm (OD_{600}) of 0.6 to 0.7 were used for chemotaxis flow assays as described in Materials and Methods. Sodium aspartate (0.1 M) in chemotaxis buffer was added (↓) at fraction 20 and removed (↑) at fraction 40. Butyric acid (31 mM) in chemotaxis buffer was added (↓) at fraction 60 and removed (↑) at fraction 80. (A) Volatile ^{3}H radioactivity; (B) total ^{3}H radioactivity (volatile plus nonvolatile).

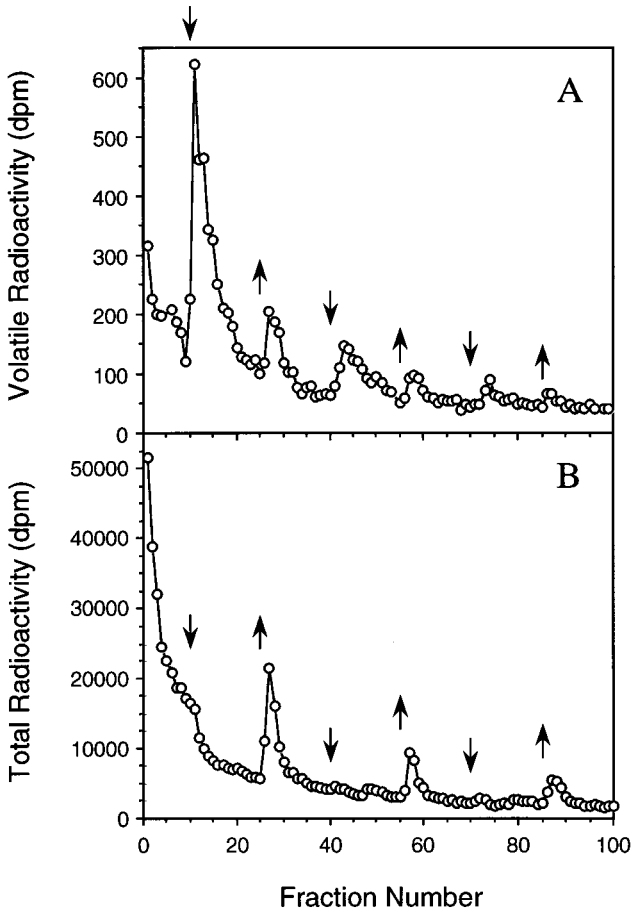

FIG. 3. Multiple exposure of *B. subtilis* OI1085 cells to 0.1 M aspartate in a chemotaxis flow assay. See the legend to Fig. 2 for a description of the experimental protocol. Sodium aspartate (0.1 M) in chemotaxis buffer was added (↓) and removed (↑) at the times indicated. (A) Volatile radioactivity; (B) total radioactivity.

The contents of the nonvolatile radioactive peaks released by *B. subtilis* in response to aspartate withdrawal or butyrate addition were analyzed by paper chromatography (Materials and Methods). The major radioactive fraction comigrated with L-methionine (data not shown). A minor component in the peak from butyrate addition comigrated with methionine sulfoxide. The major component released in response to butyrate was confirmed to be [^{3}H]methionine by amino acid analysis (Fig. 5A). Since methionine was the sole radioactive compound used to label the cells, it was possible that other amino acids were released by *B. subtilis* cells in response to the withdrawal of 0.1 M aspartate or addition of 31 mM butyrate. The experiment was repeated with *B. subtilis* cells that had been labeled with a mixture of ^{3}H-amino acids that did not contain methionine, asparagine, glutamine, or tryptophan (Fig. 5B). The results (Fig. 5C) indicated that proline, histidine, lysine, and possibly other amino acids in addition to methionine (Fig. 5A) were effluxed from *B. subtilis* in response to a repellent stimulus.

Identification of volatile radioactivity. The demonstration of amino acid efflux in response to aspartate withdrawal or butyrate addition raised the possibility that the anomalous pattern of volatile radioactivity in aspartate and butyrate chemotaxis in *B. subtilis* is an artifact. It was recognized that the [^{3}H]methionine that effluxed from cells that had been labeled with [^{3}H]methionine may break down to methanethiol during the time of vapor transfer. To address this possibility, *B. subtilis* cells were labeled with L-[^{35}S]methionine. In chemotaxis, the methyl group of methionine is transferred to γ-carboxyglutamyl residues on the chemotaxis receptors (methyl-accepting chemotaxis proteins) via *S*-adenosylmethionine (35). The radioactive ^{35}S label in [^{35}S]methionine is not transferred to the carboxymethyl ester on the receptor, and the methanol from subsequent hydrolysis of the carboxymethyl ester is therefore unlabeled. On the other hand, methanethiol derived from the breakdown of [^{35}S]methionine is radioactive. The results of perfusion of [^{35}S]methionine-labeled *B. subtilis* are shown in Fig. 6. After aspartate withdrawal, ^{35}S-labeled peaks of volatile radioactivity and total radioactivity were observed, but after aspartate addition, the volatile peak was essentially unlabeled. Compared with the results shown in Fig. 2, this suggested that methanol was released in response to aspartate addition and that the volatile radioactivity in the fractions following aspartate withdrawal included methanethiol from methionine breakdown. To test the breakdown hypothesis, L-[^{3}H-*methyl*]methionine or L-[^{35}S]methionine was dissolved in chemotaxis buffer and incubated at room temperature in a microcentrifuge tube that was placed in a scintillation vial containing 8 ml of scin-

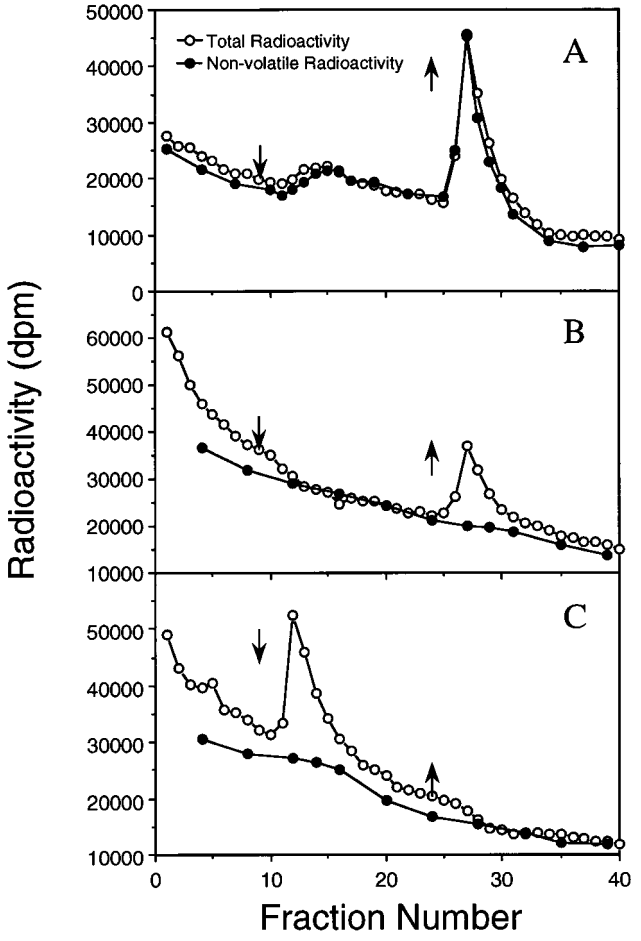

FIG. 4. Volatile and nonvolatile radioactivity released by (A) *B. subtilis* OI1085 and (B and C) *E. coli* RP437 in response to attractant removal or repellent addition. See the legend to Fig. 2 for a description of the experimental procedure. Chemoeffectors in chemotaxis buffer were added at fraction 10 (↓) and removed at fraction 25 (↑). Total radioactivity (○) was determined for each fraction, and then the volatile radioactivity was removed by heating the sample in a boiling-water bath for 10 min and the remaining nonvolatile radioactivity (●) was measured. *B. subtilis* OI1085 cells were stimulated with 0.1 M sodium aspartate (A), and *E. coli* RP437 cells were stimulated with 50 μM serine (B) or 20 mM leucine (C).

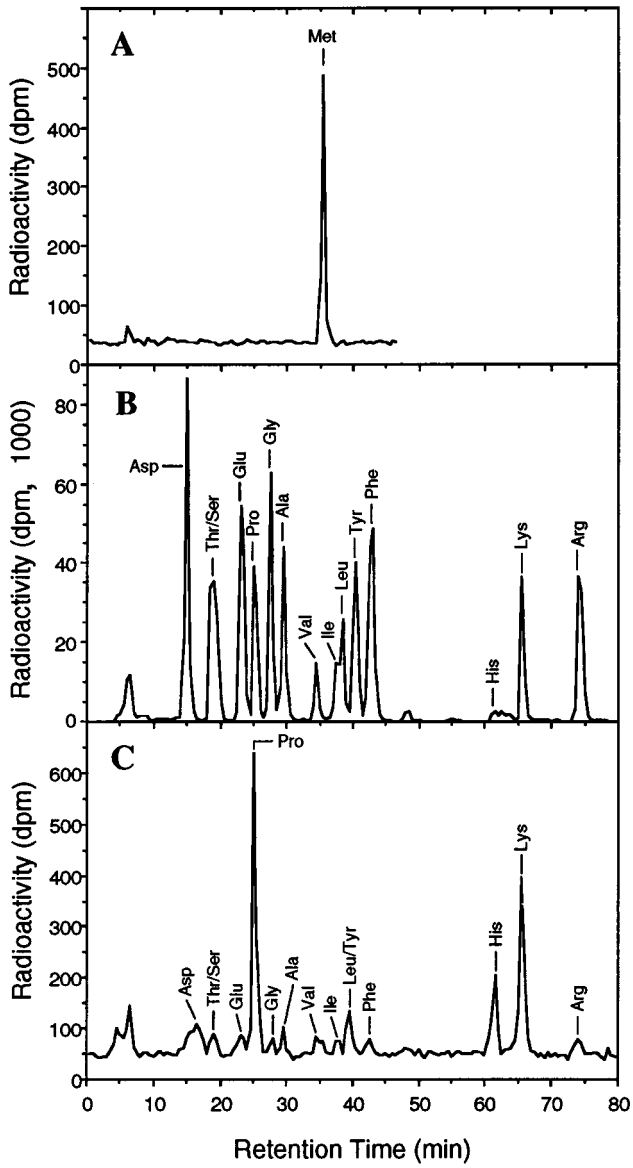

FIG. 5. Analysis of ^{3}H-amino acids effluxed from *B. subtilis* OI1085 cells in response to butyrate addition or aspartate removal. (A) The fractions containing radioactivity released from [^{3}H]methionine-labeled OI1085 cells in response to the addition of 31 mM butyric acid (shown in Fig. 4) were pooled and injected into a Biotronik 5001 amino acid analyzer as described in Materials and Methods. The profile of radioactivity eluted from the analyzer is shown. (B) Analysis of a ^{3}H-amino acid mixture used to label *B. subtilis* cells. One microliter of the ^{3}H-amino acid solution (Amersham; TRK 440; 16 to 93 Ci/mmol, 1.0 mCi/ml) was mixed with 100 μl of nonradioactive amino acid standards (2.5 μM each) and analyzed. The peaks in the radioactivity profile were identified by comparison with the profile of absorbance at 546 nm by the amino acid standards. (C) Fractions containing radioactivity released from *B. subtilis* cells labeled with the ^{3}H-amino acid mixture from part B in response to aspartate withdrawal were pooled and analyzed as in part A. The profile of eluted radioactivity is shown.

tillation cocktail (Fig. 7). Over 48 h, 0.5 and 1.8% of [^{3}H]methionine and [^{35}S]methionine, respectively, degraded to volatile radioactivity. This breakdown of methionine is sufficient to account for the increase in ^{3}H and ^{35}S radioactivity that was observed after aspartate withdrawal.

The volatile products from methionine breakdown and from the flow assay for chemotaxis were positively identified by derivitization with DNB and separation by thin-layer chromatography (Materials and Methods). When chromatographed together, the CH_3O- and CH_3S-DNB standards were not resolved with toluene-ethyl acetate at a ratio of 90:10 (22) (data not shown). After eliminating ethyl acetate from the solvent mixture, the nonradioactive CH_3O- and CH_3S-DNB spots (R_f = 0.70 to 0.71 and 0.81, respectively) were resolved. A DNB derivative of [^{3}H]methionine that had stood for 48 h comigrated with the non-radioactive CH_3S-DNB derivative (Fig. 8A). Alanine (10 mM) was added as a chemoattractant in a flow assay of *B. subtilis* methylation in vivo. Two pools were formed from fractions that contained volatile radioactivity released in response to alanine addition and to alanine withdrawal. Each pool was immediately derivatized with DNB. The adducts from both pools comigrated with the CH_3O-DNB standard (R_f = 0.70), indicating that methanol was released by *B. subtilis* in response to alanine addition or withdrawal (Fig. 8B and C) (1). The amount of methanol released after alanine withdrawal (Fig. 8C) was three- to fourfold less than the amount released after alanine addition (Fig. 8B). A basal level of methanol was released by unstimulated cells (data not

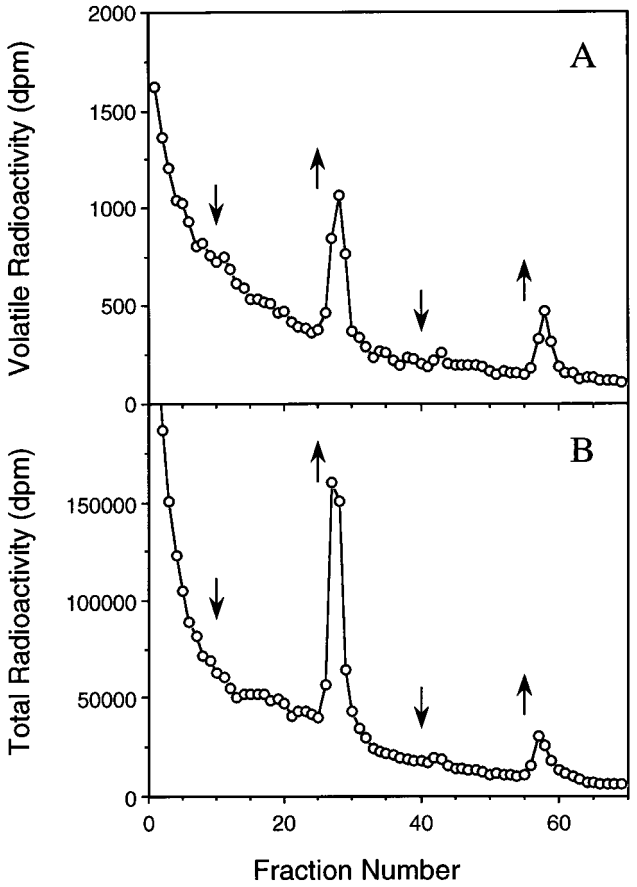

FIG. 6. Volatile and nonvolatile radioactivity released by [^{35}S]methionine-labeled *B. subtilis* OI1085 cells in response to 0.1 M aspartate. Cells were grown in Luria-Bertani broth at 35°C and harvested at an OD_{600} of 0.8. Approximately 10^9 cells were washed with chemotaxis buffer, labeled with 20 μl of [^{35}S]methionine (DuPont NEN; 1,052 Ci/mmol, 10.29 mCi/ml), and used for a chemotaxis flow assay as described in Materials and Methods. Arrows indicate addition (↓) and removal (↑) of aspartate. (A) Volatile radioactivity after a 12-h vapor transfer; (B) total radioactivity.

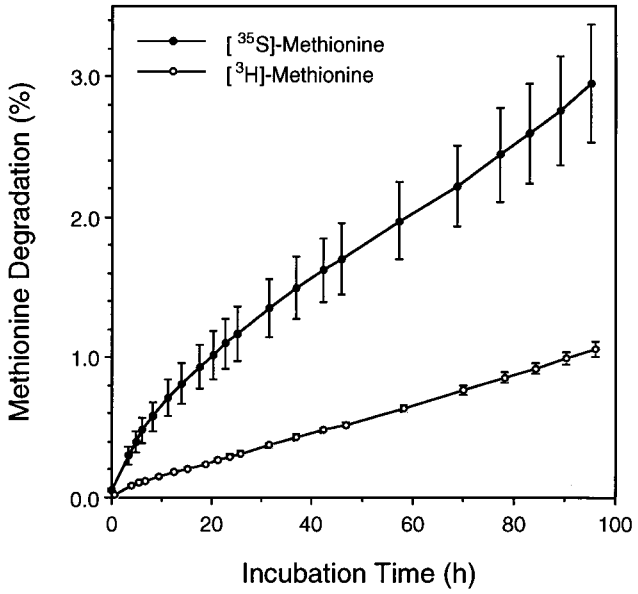

FIG. 7. Degradation of [^{35}S]- and [^{3}H]methionine at room temperature. One hundred microliters of chemotaxis buffer containing approximately 1 μl of [^{3}H]- or [^{35}S]methionine was placed in a microfuge tube, and volatile counts were allowed to transfer to a 7-ml scintillation cocktail in a 20-ml scintillation vial. Each datum point is the average of four determinations. Error bars represent standard deviations.

shown). In an aerotaxis flow assay in *B. subtilis*, the volatile radioactivity released in response to the addition or withdrawal of oxygen was derivatized and identified as methanol (38).

Relationship between chemotaxis and nonvolatile radioactivity. The *B. subtilis cheR* mutant OI1100 is deficient in the methyltransferase activity required for chemotaxis. A comparison of flow assays for *B. subtilis* OI1085 (wild type) and OI1100 revealed that the *cheR* mutant lacks the volatile peak after addition of aspartate (Fig. 9). The small increase in volatile radioactivity in the *cheR* mutant following removal of aspartate is assumed to derive from methionine efflux (Fig. 9B) and degradation. In the *cheR* mutant, the relative increase in non-volatile radioactivity after aspartate withdrawal compared with the baseline value was not significantly different from that in the wild-type cells (Fig. 9B). This indicated that the amino acid efflux that follows 0.1 M aspartate withdrawal is not dependent on chemotaxis.

When lower concentrations of chemoeffectors were used, we did not observe the distinctive pattern of amino acid efflux that was observed with 0.1 M aspartate. In flow assays of aerotaxis and of chemotaxis to 10 mM alanine and to 10 mM proline, there was no peak of nonvolatile radioactivity after a repellent stimulus (data not shown). A reduced level of non-volatile efflux was observed, but it was not associated with a positive or negative stimulus. The volatile radioactivity in response to 10 mM alanine showed peaks after both addition and withdrawal of alanine. Volatile radioactivity was released after 10 mM proline addition, but not after withdrawal (data not shown).

Colligative properties and amino acid efflux. To investigate the basis for the observed amino acid efflux in *B. subtilis*, 0.1 M aspartate was replaced in the flow assays by 0.1 M NaCl, 0.2 M sucrose, or 0.2 M xylitol. Each changed the osmolarity of the perfusion buffer from 40 to 240 mOsm, as did 0.1 M aspartate, but sucrose and xylitol did not change the ionic strength. A profile of the volatile and total radioactivity released by *B. subtilis* in response to the addition and withdrawal of 0.1 M NaCl is shown in Fig. 10. The pattern of volatile radioactivity released resembles the pattern for chemotaxis (compare Fig. 10 and 2). Total radioactivity released (Fig. 10B) was similar to that observed previously for amino acid efflux. Removal of 0.2 M sucrose or 0.2 M xylitol also elicited an efflux of amino acids (data not shown), indicating that the decrease in osmolarity is the stimulus for amino acid release. Control experiments with Formalin-treated cells ruled out nonspecific adsorption to cell membranes or to the filter as the source of amino acids that were released into the perfusate. The transport of methionine and other amino acids was unaffected by 0.1 M aspartate or 0.1 M NaCl over a 90-s interval (data not shown).

Behavioral studies of the responses of *B. subtilis* OI1085 to osmotic shifts revealed the following. Addition of NaCl to a final concentration of 0.1 M or of xylitol to a final concentration of 0.2 M (200 mosM upshift) caused a tumbling response of 18 ± 2 s and 16 ± 2 s, respectively. There was a direct correlation between the magnitude of the upshift in the range of 200 to 800 mosM and the adaptation time (16 to 75 s) (data not shown). An increase in osmolarity to more than 1 osM caused paralysis of *B. subtilis* cells. The cells resumed motility only when osmolarity was decreased by addition of distilled water. An osmotic downshift in which the osmotic strength of

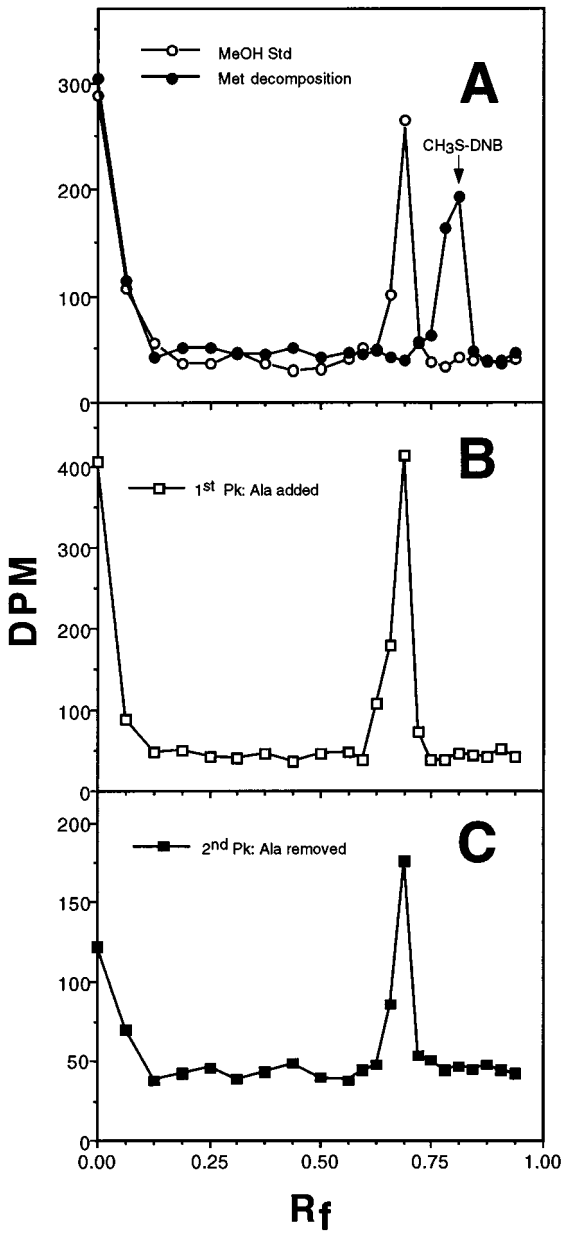

FIG. 8. Thin-layer chromatographic separation of DNB derivatives of flow assay fractions. See Materials and Methods for a description of experimental protocol. (A) $^{14}CH_3O$-DNB (derivatized [^{14}C]methanol) (○) and derivatized radioactivity from the 48-h decomposition of [^{3}H]methionine (●). The arrow represents the migration of nonradioactive CH_3S-DNB (derivatized methanethiol). (B and C) Derivatized pooled fractions of the peaks after alanine addition (□) and alanine withdrawal (■), respectively.

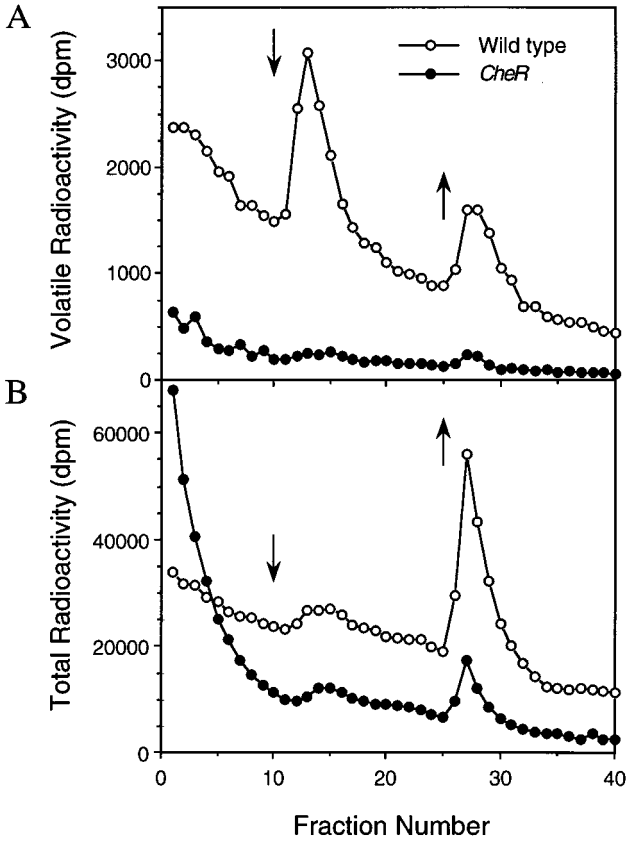

FIG. 9. Comparison of volatile and nonvolatile radioactivity released by wild-type (OI1085) and *cheR* mutant (OI1100) *B. subtilis* cells. See the legend to Fig. 2 for a description of the experimental procedure. (A) Volatile and (B) total radioactivity released in the flow assays of wild-type OI1085 (○) and *cheR* mutant OI1100 (●) *B. subtilis* cells. Arrows indicate addition (↓) and removal (↑) of 0.1 M aspartate in chemotaxis buffer.

a medium containing from 200 to 800 mosM NaCl or xylitol was lowered by dilution 1:1 with deionized water did not cause a tumbling response, whereas a downshift from 40 mosM (chemotaxis buffer) to 20 mosM caused a tumbling response of 14 ± 4 s.

DISCUSSION

Aspartate concentrations as high as 0.1 M have been used in previous studies of chemotaxis in *B. subtilis* (9, 29, 31). The withdrawal of 0.1 M aspartate in the flow assay of chemotaxis presents the bacteria with a hypoosmotic shift of a magnitude that produces rapid extrusion of small molecules in *E. coli* (3, 24, 33). When *E. coli* cells are transferred to a hypertonic medium, the bacteria accumulate K^+ and synthesize or take up glutamate to maintain turgor (8, 14). Proline, glycine betaine, and trehalose can also be accumulated as protectants against hyperosmotic stress (21, 27). If the *E. coli* cells are returned to a medium of lower osmolarity, the bacteria must rapidly excrete the excess small molecules into the medium. This is believed to involve large stretch-activated channels (3). In *B. subtilis*, K^+, proline, and glycine betaine appear to be the most important osmoprotectants (4, 36).

In this study, the withdrawal of 0.1 M aspartate produced an efflux of nonvolatile radioactivity that included methionine, proline, lysine, and histidine. Lesser amounts of leucine and tyrosine were effluxed. The rapid release of nonvolatile radioactivity shown in Fig. 2 is consistent with efflux mediated by large stretch receptor channels and is probably too fast for carrier-mediated efflux (3). A similar efflux of nonvolatile radioactivity was observed after the withdrawal of 0.1 M NaCl (Fig. 10), 0.2 M xylitol, or 0.2 M sucrose, indicating that the bacteria are responding to colligative changes in the medium. There was little, if any, efflux of glutamate, as is observed in *E. coli*. Methionine and histidine are not important osmoprotectants in *E. coli* or *B. subtilis*. However, intracellular pools of specific amino acids in *E. coli* may be elevated above normal

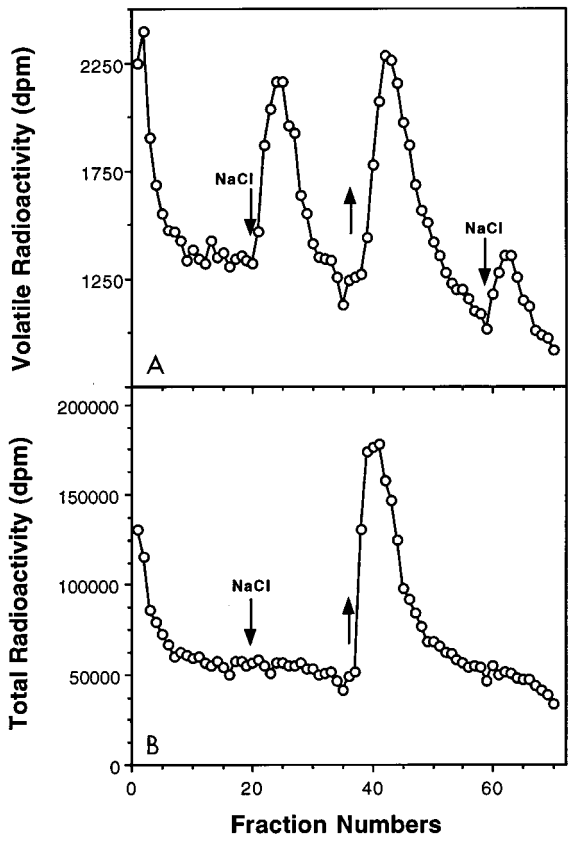

FIG. 10. Volatile and total radioactivity released by wild-type *B. subtilis* OI1085 in response to 0.1 M NaCl addition and removal. See the legend to Fig. 2 for a description of the experimental procedure. NaCl (0.1 M) in chemotaxis buffer was added (↓) and removed (↑) as shown. (A) Volatile ^{3}H radioactivity after 30 h of vapor transfer. (B) Total ^{3}H radioactivity.

levels when the amino acids are present in the external medium. The efflux of methionine that was observed in *B. subtilis* may reflect a high concentration of free methionine in the cells, since exogenous methionine was present before the osmotic downshift.

An unexpected finding in this study was that the degradation of effluxed methionine during the vapor transfer phase of the flow assay can be a major contributor to the repellent-stimulated release of volatile ^{3}H radioactivity by *B. subtilis*. The degradation of methionine in the flow assay has been reported (12), but the overall result was an increase in baseline radioactivity throughout the flow assay profile and not the formation of a spurious peak. The volatile product of methionine breakdown was identified as methanethiol by labeling *B. subtilis* with [^{35}S]methionine (Fig. 6) and by derivatizing the volatile peak with DNB (Fig. 8). The rate of methionine breakdown (Fig. 7) was sufficient to account for the methanethiol that was observed.

Under experimental conditions in which methanethiol formation was minimized, two peaks of volatile radioactivity were observed during *B. subtilis* chemotaxis, as reported previously by Thoelke et al. (30). No peak of amino acid efflux was observed after the addition and withdrawal of 10 mM alanine, 10 mM proline, or oxygen (data not shown). Ordal and colleagues (1, 10) previously positively identified methanol as the product of demethylation of the methyl-accepting chemotaxis proteins in *B. subtilis*. This study identified [^{3}H]methanol in both peaks of volatile radioactivity that are associated with attractant addition and withdrawal (Fig. 8). The amount of methanol released was much greater after alanine addition than after alanine withdrawal. The methanol release is clearly related to chemotaxis: methanol release but not methanethiol formation was abolished in a *cheR* mutant that was deficient in the methyltransferase protein for chemotaxis.

B. subtilis demonstrated a behavioral response to temporal changes in osmolarity, i.e., osmotaxis. Osmotic upshifts (200 to 800 mosM) caused a negative taxis in *B. subtilis*, indicating a repellent response to high osmolarity. In contrast to *E. coli* (15), *B. subtilis* showed only one type of behavioral response to osmotic upshift, a tumbling response followed by adaptation. Large osmotic upshifts (400 to 800 mosM) that caused a prolonged (up to 15 min) stopping and pseudotumbling response in *E. coli* caused a relatively short (up to 75 s) tumbling response in *B. subtilis*. It appears likely that *B. subtilis*, like *E. coli* (16), is repelled by both high and low osmolarity and attracted by an optimal osmolarity, but this has not been confirmed in a spatial osmotic gradient. A transient release of volatile ^{3}H radioactivity observed following the osmotic upshift (Fig. 10) suggests that osmotaxis in *B. subtilis* might be methylation dependent, similar to the methylation-dependent aerotactic response in this bacterium (38).

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