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Effect of Methionine on Chemotaxis by *Bacillus subtilis*

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Bacillus subtilis, like *Escherichia coli* and *Salmonella typhimurium,* carries out chemotaxis by modulating the relative frequency of smooth swimming and tumbling. Like these enteric bacteria, methionine auxotrophs starved for methionine show an abnormally long period of smooth swimming after addition of attractant. This "hypersensitive" state requires an hour of starvation for its genesis, which can be hastened by including alanine, a strong attractant, in starvation medium. Susceptibility to repellent, which causes transient tumbling when added, if anything, increases slightly by starvation for methionine. The results are interpreted by postulating the existence of a methionine-derived structure that hastens recovery of attractant-stimulated bacteria back to normal.

Chemotaxis is the process by which an organism swims to higher concentrations of attractant or lower concentrations of repellent. Berg and Brown (5) found that chemotaxis of peritrichous enteric bacteria, which normally swim and tumble (thrash about) in an endless series, occurs by suppression of tumbles when the bacteria are swimming in the favorable direction (up gradient of attractant or down gradient of repellent). Macnab and Koshland (7) found that chemotaxis could be studied by direct behavioral observation: attractant added to bacteria promotes transient swimming; repellent promotes transient tumbling. The question is, of course, how do attractants cause swimming or repellents cause tumbling?

One means of investigating this question focuses on discovering what controls frequency of tumbling (and swimming). One clue to this puzzle arose early in the modern work on chemotaxis when Adler and Dahl (1) found that a methionine auxotroph of *Escherichia coli* could not carry out chemotaxis unless methionine was present in the chemotaxis buffer and that such bacteria tumbled infrequently. Aswad and Koshland (3, 4) also found the basis of the requirement for chemotaxis in wild-type *Salmonella typhimurium* to be the need for methionine or its derivative for ability to tumble. It should be noted, however, that a mutant ST4, a methionine auxotroph showing an abnormally high natural tumbling frequency in the presence of methionine, continued to tumble in absence of methionine (3). However, after addition of attractant, it swam much longer when starved for methionine rather than for histidine or tryptophan, with methionine present (3). Armstrong (2), whose conclusions were confirmed by Aswad and Koshland (4), has indicated that the requirement for methionine really reflects the need for *S*-adenosylmethionine, a methylating agent, although *S*-adenosylmethionine itself does not directly affect tumbling frequency (4).

Therefore, the question likely is, what becomes methylated and what role does this methylation play in regulating tumbling frequency? To investigate any requirement for methionine in ability of *Bacillus subtilis* to tumble, methionine auxotrophs of strains differing in natural tumbling frequency were selected and studied. Results show that methionine is needed for normal recovery from swimming induced by adding attractant, much like *S. typhimurium* strain ST4 (4). However, the results tentatively suggest that methionine is not required for tumbling per se by *B. subtilis.*

MATERIALS AND METHODS

Bacteria. Strains OI8, which mostly swims, and OI151, which mostly tumbles, have been described (9). Strain OI363 is a methionine auxotroph of OI8, and OI152 is a methionine auxotroph of OI151. Both were selected by mutagenesis followed by staphcillin selections for auxotrophs, as described below.

Media. Nutrient broth containing $CaCl_2$, $MgCl_2$, and $MnCl_2$, tryptone broth, minimal medium, and chemotaxis buffer were used as described (9), except that 5 mM sorbitol replaced glycerol and sodium lactate in chemotaxis buffer. Mutagenesis buffer contained 0.1 M potassium phosphate (pH 7), 2 mM potassium glutamate, and 0.2 mM ethylenediaminetetraacetic acid. Minimal plates contained the ingredients of minimal medium except that potassium phosphate was 10 mM (not 50 mM), required amino

acids were 0.1 mM (not 0.3 mM), and agar concentration was 1.5%.

Microscopy observation of bacteria. One-tenth milliliter of bacteria was inoculated from a stationary-phase tryptone broth culture into 5 ml of minimal medium and grown into midexponential phase. For some experiments, bacteria were filtered on Millipore filters, washed, and resuspended at 18 Klett units (filter 66) per ml. For other experiments, the bacteria were centrifuged, washed twice, and resuspended at around 5 to 10 Klett units in medium that was the same, except that it was lacking or limited for a certain amino acid (starvation conditions). In the latter case, these limiting concentrations were 2.3, 15, and 15 μM for methionine, isoleucine, and valine, respectively. For observation, bacteria were placed on microscope slides in 9-μl drops into which 1 μl of reagent was injected, as described (9, 9a). Where possible, techniques were used so that experiments were carried out blind; e.g., labels were covered on flasks or tubes from which bacterial samples or reagent (attractant, repellent, or buffer) was taken.

Capillary assays. The method of Ordal and Goldman (9) was used, with modifications. Bacteria were diluted to 0.0002 optical density units at 600 nm. A 1-μl capillary containing attractant was inserted into the suspension at 37 C. Attractant diffused from the capillary into the "pond" containing bacteria to create a gradient, causing influx of bacteria. After 0.5 h, the tube was withdrawn and rinsed, and its contents were extruded into buffer and plated. Colonies were counted the next day.

Mutagenesis and selection of methionine auxotrophs. The method was based on that of Ordal and Adler (8). Bacteria were grown in nutrient broth to 180 Klett units, washed twice, and suspended at 5×10^7 bacteria/ml in mutagenesis buffer at room temperature (23 C). Three percent ethyl methane sulfonate was added. After an hour, the bacteria were washed and grown overnight in tryptone broth. To select methionine auxotrophs, bacteria were inoculated into minimal medium with methionine, grown to midexponential phase, washed three times, put into medium without methionine, and grown for an hour. Then 5 mg of staphcillin per ml was added and growth was continued for an hour. The bacteria were washed three times and grown overnight in tryptone broth. The sequence was repeated once and the bacteria were plated on minimal plates containing methionine and replica plated onto pairs of the same type of plates, one lacking and the second having methionine.

RESULTS

Effect of methionine in the capillary assay. The capillary assay, the traditional assay of chemotaxis, measures ability of bacteria to respond to spatial gradients of attractant. To test whether methionine auxotrophs of *B. subtilis*, deprived of methionine, can still carry out chemotaxis in this assay, OI363 was assayed for asparagine and mannitol taxes in presence and in absence of methionine. Methionine, far from being required for chemotaxis, slightly inhibited chemotaxis to both asparagine and mannitol (Table 1).

Response of methionine auxotroph to temporal gradients. To determine whether attractant added to methionine auxotrophs of *B. subtilis* causes prolonged swimming, as it does for *S. typhimurium* (3) and *E. coli* (11), various concentrations of asparagine were added to strain OI152. The result is shown in Table 2, which shows that lack of methionine does result in prolonged swimming, compared with presence of methionine. Motility of the bacteria in presence of methionine was the same as in its absence.

Starvation for methionine. To see whether starvation for methionine in growth conditions, which might be expected to reduce endogenous levels of methionine, would lead to longer swimming on addition of attractant, OI152 was starved in growth medium for methionine. Figure 1 shows the results when bacteria are placed in growth medium with a small amount of methionine as the limiting amino acid. It

TABLE 1. *Capillary assay of chemotaxis in presence or absence of methionine*[a]

Concn of attractant, μM	No. of bacteria in capillary	
	With methionine	No addition of methionine
Asparagine		
1	139	186
10	226	475
100	1,084	1,525
Mannitol		
0.1	68	171
1	209	305
10	657	463
100	606	693
No attractant	17	33

[a] See Materials and Methods. Methionine concentration was 0.1 mM.

TABLE 2. *Swimming induced by asparagine by methionine auxotroph*[a]

Concn (mM)	Methionine present[b] (s)	Buffer only (s)
0.01	7	10
0.1	20	48
1	35	>300
10	102	>300

[a] Experiment blind. See Materials and Methods.
[b] 0.1 mM methionine preincubated for several minutes.

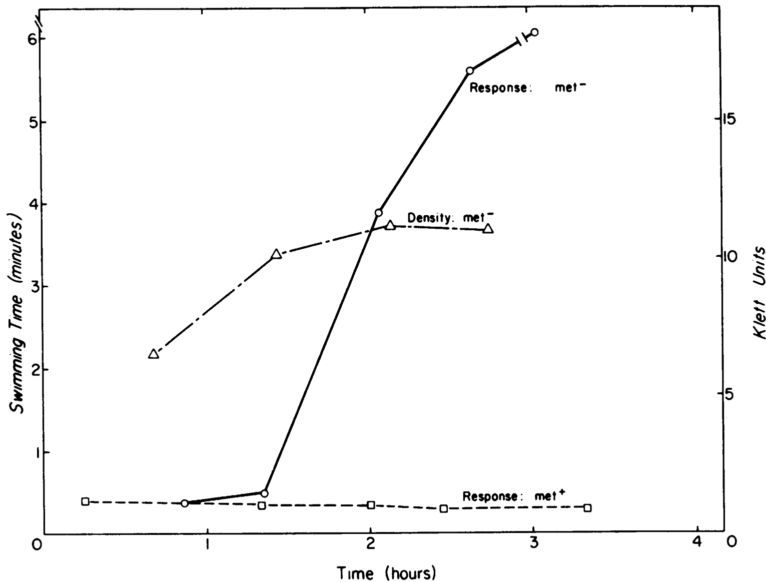

FIG. 1. *Response of methionine auxotroph and prototrophic parent to 32 μM asparagine. Strains OI151 and OI152 were grown in minimal medium and at zero time were transferred to low-methionine medium and shaken at 37 C. On the abscissa is graphed time of incubation, and on the ordinate, time for the bacteria to return to approximately normal tumbling frequency or density of the methionine auxotroph. Symbols: (○) Swimming response of methionine auxotroph, (□) swimming response of prototrophic parent, (△) density of methionine auxotroph.*

can be seen that starvation for methionine leads to prolonged swimming by the methionine auxotroph. However, starvation for isoleucine and valine does not (data not shown; however, see Fig. 2), and incubation by the methionine prototrophic parent in limited methionine medium likewise does not (Fig. 1).

Two further observations should be noted. First, the bacteria did not become smooth swimming merely through starvation (however, see below). When the bacteria were transferred from shaking culture at 37 C to a microscope slide at room temperature, they sometimes, particularly during methionine starvation, briefly swam smoothly, but then they tumbled normally. Thus, these bacteria are similar to strain ST4 *(S. typhimurium)* of Aswad and Koshland (3). Moreover, even OI363, a *met* auxotroph of strain OI8, which mostly swims, does not become completely smooth swimming from methionine starvation in growth medium. Second, the effect of prolongation of swimming on addition of 32 μM asparagine worked much better on cells incubated at 37 C, compared with those at room temperature.

To assess any requirement for protein synthesis in genesis of bacteria that were "hypersensitive" to low concentrations of attractant, OI152 was starved on isoleucine and valine; then the culture was split, and one half was starved for methionine, as well as for isoleucine and valine, and the other half was starved for methionine only. Fig. 2 shows that the presence of the required amino acids, isoleucine and valine, was needed to make the bacteria hypersensitive.

The interpretation of this experiment is ambiguous. Protein synthesis may be necessary to deplete methionine arising from proteolysis or, alternatively, may be required for synthesis of a structure, which "favors" swimming, to replace the "methionine-linked" structure, which favors tumbling. The former is the most economical hypothesis. However, 2 mM phenyl methyl sulfonyl fluoride, a protease inhibitor (10), when added to OI152 starved for methionine, had no effect on length of asparagine-induced swimming. Because it caused about 10 s of tumbling (i.e., it is a weak repellent), it probably did enter the bacterium (9, 9a).

Incubation in attractant. Alanine is a good attractant for *B. subtilis,* with a threshold of 3 nM, and is sensed by a different receptor from asparagine (G. W. Ordal, K. Gibson, and D. Villani, in progress). Springer et al. (11) have found that incubation of methionine auxotroph tumbling mutants of *E. coli* in attractant hastens the onset of conversion from mostly tumbling to smooth swimming. To discover

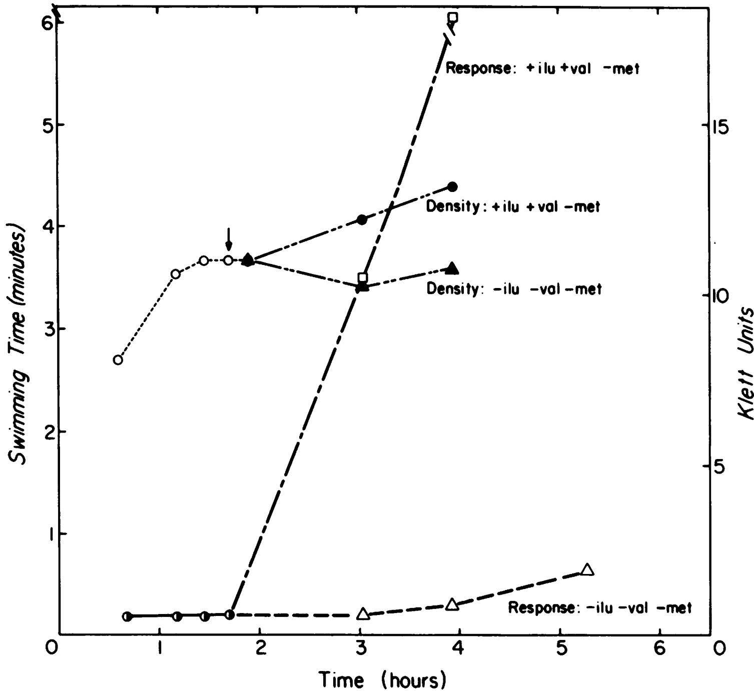

FIG. 2. *Effect of starvation for isoleucine and valine and for methionine. Strain OI152 was grown in minimal medium and then washed and put in medium with low isoleucine and valine. At the time indicated by the arrow, the culture was divided, washed, and put into medium lacking methionine but having or lacking isoleucine and valine. Symbols: (◑) Response and (○) density of culture in presence of methionine but low in isoleucine and valine (before splitting of culture), (□) response and (●) density of culture in absence of methionine but presence of isoleucine and valine, (△) response and (▲) density of culture in absence of methionine and of isoleucine and valine.*

whether attractant has the same effect for *B. subtilis*, OI152 was supplemented with 1 mM alanine 11 min before harvest. The culture was divided and washed in presence or absence of alanine and was then put into the same medium, but lacking methionine, in presence or absence of alanine. Results of observing the duration of swimming after stimulation with 32 μM asparagine as a function of time are presented in Fig. 3. It can be seen that presence of alanine hastens onset of the hypersensitive state. It should be emphasized that alanine was not added as a chemotactic stimulus: the bacteria had adapted to it much earlier during growth in presence of methionine. Moreover, one should remember that starvation medium included 0.3 mM isoleucine, which has a threshold of 0.6 μM, and 0.3 μM valine, which has a threshold of 0.13 μM. These have receptors distinct from the alanine and asparagine receptors (G. W. Ordal, K. Gibson, and D. Villani, in progress). Finally, it is noteworthy that 1 h after onset of incubation in presence of alanine an appreciable fraction of bacteria become smooth swimming, even without attractant having been added, a result not otherwise seen.

Return to normal state. Can bacteria starved for methionine, so that they are hypersensitive to a low concentration of attractant, return to normal after methionine is reintroduced? Fig. 4 shows that addition of 0.1 mM methionine did cause OI152 to regain normal sensitivity to attractant. However, return to normal was far from instantaneous; possibly, it took too long to be accounted for by the time necessary for buildup of an intermediary metabolite. The need for a structure to be replaced—a "swimming favoring" one by a "tumbling favoring" one—might account for such a result. To explore this, 0.1 ml of methionine-starved OI152 was added to 0.02 ml of 1 mM methionine or 0.02 ml of 1 mM methionine plus 0.02 ml of chloramphenicol (2 mg/ml). After 200 s, 0.02 ml of chloramphenicol (2 mg/ml) was added to the bacteria not given the antibiotic previously. Then, in a blind experiment, the bacteria were placed on a microscope slide and given 32 μM asparagine about a minute later. The result was that the bacteria to which chlor-

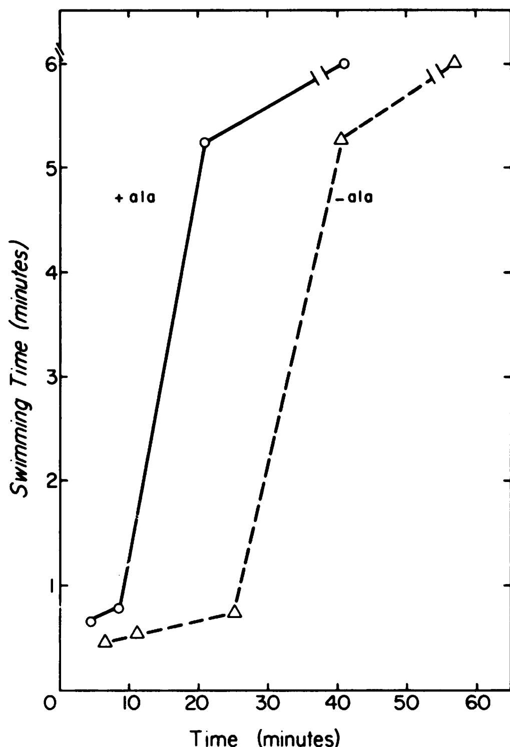

FIG. 3. *Effect of incubation in alanine. See text for procedure. Alanine concentration was 1 mM. Symbols: (○) With alanine, (△) without alanine.*

amphenicol was added simultaneously with methionine swam for 144 ± 24 s, whereas the bacteria to which chloramphenicol was added later swam for 77 ± 17 s, which is significantly different at the 0.02 level by the *t* test. This result implies that return to normal tumbling capability occurs faster when protein synthesis can occur.

Several further points should be made, however. First, the bacteria in absence of methionine swam for 312 s when given 32 μM asparagine. Second, chloramphenicol by itself caused "hypersensitive" bacteria to return to close to normal (possibly by allowing buildup of methionine, see above), although somewhat more slowly than after addition of methionine (bacteria swam for 2 min after addition of 32 μM asparagine added 4.5 min after chloramphenicol). Third, chloramphenicol itself causes brief tumbling (i.e., it is a weak repellent). However, asparagine added as a function of time after chloramphenicol evoked swims of the same duration: i.e., there were no changing "after effects." In sum, these data suggest that protein synthesis aids the action of methionine in reconverting cells from hypersensitive to normal but stop short of defining a requirement, since the bacteria eventually reconvert in presence of chloramphenicol alone, of their own accord.

Effect of methionine analogues. The methionine analogue norleucine, in which a methylene group replaces the sulfur, was incubated with OI151 in conditions of methionine starvation. Table 3 shows that incubation in presence of the analogue hastened genesis of the hypersensitive condition. It may be worth noting that the bacteria increased in density, albeit slowly, in presence of norleucine, compared with bacterial density in its absence (Table 3). The methionine analogue, cycloleucine (1-amino-1-cyclopentane-carboxylic acid), on the other hand, despite being a better inhibitor of *S*-adenosylmethionine synthetase than norleucine (6), did not affect onset of the hypersensitive state at 1 mM. At 50 mM, however, cycloleucine actually prevented genesis of the hypersensitive state in

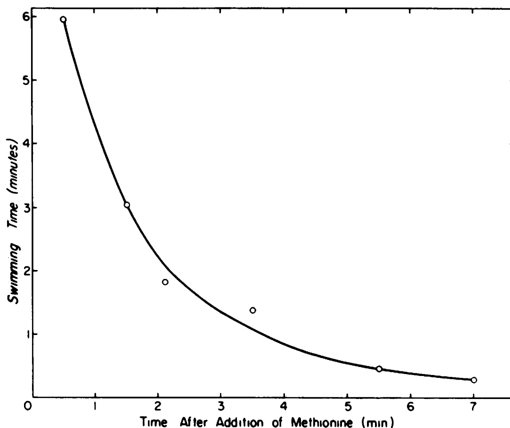

FIG. 4. *Recovery from hypersensitive state. One-tenth millimolar methionine was added to strain OI152 previously starved for methionine at 37 C. At the indicated times, 9-µl aliquots were removed and placed on a microscope slide at room temperature and soon afterward, at the indicated times, were injected with 32 µM asparagine.*

TABLE 3. *Effect of norleucine on responsiveness of methionine auxotroph*

Response to 32 µM asparagine (s)			Density of culture (Klett units)		
Time[a] (min)	Norleucine present	No addition	Time[a] (min)	Norleucine present	No addition
22	248	23	15	9.2	9.2
75	>300	265	70	12.5	7.3
177	>300	>300	184	15.3	10.1

[a] After culture placed at 37 C, with shaking, in growth medium lacking methionine.

strain OI363. In fact, these bacteria incubated in growth medium lacking methionine at room temperature for an hour tumbled more when 50 mM cycloleucine was present. Control experiments (data not shown) showed that (i) cycloleucine is taken up by the bacteria, (ii) 50 mM cycloleucine reduces internal *S*-adenosylmethionine levels, and (iii) presence of 50 mM cycloleucine does not affect duration of swimming caused by 32 μM asparagine. Unlike norleucine, bacteria in cycloleucine did not increase in density faster than the control bacteria (no cycloleucine present). It should be noted that norleucine and cycloleucine are themselves attractants, 1 mM norleucine causing OI151 to swim 39 s and 1 mM cycloleucine causing swimming for 12 s.

Effect on repellents. Repellents of *B. subtilis* are thought to act by a different mechanism than attractants (9a), although in enteric bacteria both are thought to act by a similar mechanism. How is sensitivity of bacteria to repellent affected by methionine starvation? For this purpose, OI363, which is a methionine auxotroph of OI8, which mostly swims, was starved for methionine analyzed to determine, as a function of time, the minimum concentration (threshold) of the uncoupler trifluoromethyoxycarbonylcyanidephenylhydrazone (FCCP), a repellent, to cause tumbling. For comparison, the bacteria were starved for isoleucine and valine. Fig. 5 shows thresholds for FCCP of bacteria starved for methionine or isoleucine and valine, and corresponding growth curves.

Two conclusions may be drawn from this data. First, starvation for methionine does not decrease sensitivity to repellents: if anything, it increases sensitivity. Second, these bacteria are much more sensitive to FCCP than bacteria grown in nutrient broth and transferred to chemotaxis buffer, for which the threshold is 10 nM (9a).

In a further experiment, OI363 was grown on minimal medium and transferred to two flasks of medium that was the same except that methionine was entirely lacking. One flask contained norleucine; the other did not. After 40 min, the bacteria in medium with norleucine had a threshold for FCCP of 1.8 nM; bacteria without norleucine had a FCCP threshold of 1.0 nM. The bacteria in norleucine had a higher natural tumbling frequency. After 2.5 h, bacteria in both flasks had a threshold of 3.2 nM. Incubation of the norleucine-free bacteria in 0.1 mM methionine for 0.5 h longer did not change this threshold. In a similar experiment, incuba-

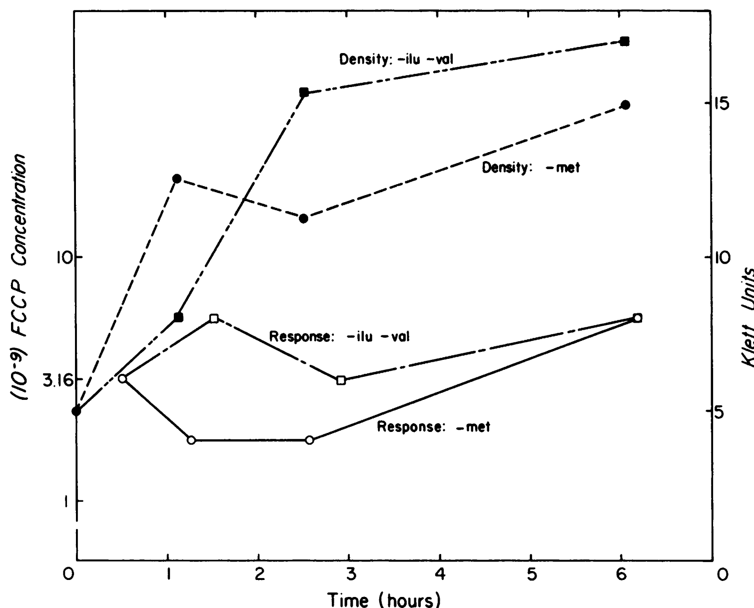

FIG. 5. *Effect of starvation for methionine or for isoleucine and valine on threshold of response to FCCP. Strain OI363 was grown in minimal medium, washed, and split in two parts. One part was then grown in medium having low concentrations of isoleucine and valine, but ample methionine, and the other was then grown in medium having low concentration of methionine but ample isoleucine and valine. At different times, each culture was examined for threshold of response to FCCP in a blind test at 1.8-fold intervals, as described previously (9a). Symbols: (○) Response and (●) density of methionine-starved culture, (□) response and (■) density of isoleucine- and valine-starved culture.*

tion of OI363 in 1 mM cycloleucine for about 2 h had no effect on threshold of FCCP, which was 3.2 nM.

Finally, a culture of OI363 was grown on minimal medium, given 1 mM alanine, and after 10 min (well after adaptation) was split, washed twice, and put in similar medium with 10 μM ethylenediaminetetraacetic acid, with the following differences: (i) no isoleucine and valine, (ii) no methionine, and (iii) no methionine with 1 mM alanine. After 2 h at 37 C, the bacteria were tested for thresholds for FCCP, which were (i) 3.2 nM, (ii) 1.8 nM, and (iii) 1.0 nM, respectively.

DISCUSSION

Although *B. subtilis,* being gram positive, is phylogenetically distinct from enteric bacteria like *E. coli* and *S. typhimurium,* which are gram negative, behavior of all these organisms is affected by deprivation of methionine (1, 3, 4, 11). In particular, suspension of methionine auxotrophs of these bacteria in buffer lacking methionine causes prolonged swimming on stimulation by attractant (4, 11). Presence of methionine prevents this abnormal response.

In this article, we have shown that a "hypersensitive" state develops as the result of starvation for methionine. In this condition, the bacteria swim for over 6 min after addition of 32 μM asparagine, which evokes only 10 to 20 s of swimming from unstarved bacteria. The main question is, why does it take so long? This condition requires an hour after onset of starvation to be complete. To return to normal takes a shorter time, about 7 min, and is faster when protein synthesis is allowed rather than prevented.

It does not seem plausible that variation in the level of an intermediary metabolite can account for these results. Rather, they imply, it seems to me, that a structure, partly derived from methionine, exists that promotes recovery from attractant-induced swimming back to normal. This structure is probably itself partly destroyed by "signals" from attractant, since presence of alanine hastens onset of the hypersensitive state (Fig. 3). Although norleucine can, to a slight extent, satisfy the methionine requirement for growth (the density of the culture increases somewhat, Table 3), it promotes genesis of the hypersensitive state faster than mere starvation for methionine. Armstrong (2) and Aswad and Koshland (4) have indicated that the methionine requirement really reflects a requirement for *S*-adenosylmethionine, a methylating agent. In this context it is paradoxical that cycloleucine, which inhibits chemotaxis in *S. typhimurium* (4) and is a better inhibitor of *S*-adenosylmethionine synthetase than norleucine (6), did not similarly hasten onset of the hypersensitive state, although it may be that norleucine promotes hypersensitivity by virtue of acting as an attractant, like alanine. In fact, very high concentrations (50 mM) of cycloleucine prevented genesis of hypersensitivity. On balance, however, these findings suggest existence of a structure which, when methylated, hastens recovery from attractant-induced swimming.

Is methionine, or a derivative, required for tumbling itself, not merely to aid in recovery from swimming caused by attractant? The answer to this question is tentatively no. First, the bacteria were never found to swim freely, no matter how long they were starved for methionine, unless alanine was present. Second, when OI363, which mostly swims, is starved for methionine, presence of norleucine makes the bacteria tumble more than absence of norleucine, despite their greater sensitivity to attractant.

Third, if the bacteria starved for methionine become more disposed to swim and less to tumble, then they might be expected to become less sensitive to repellent. The results show, however, that starvation for methionine did not decrease sensitivity to repellent – if anything, it increased slightly. Moreover, norleucine had little effect. Presence of alanine did not decrease sensitivity to a FCCP either, although the bacteria swam somewhat more smoothly in its presence. It is noteworthy that repellents probably act by a different mechanism, in part, from attractants, unlike repellents of enteric bacteria (9a).

Aswad and Koshland (3) have made a similar proposal – that methionine affords an environment favoring tumbling but is not an obligate requirement. The results of my experiments largely fit this proposal.

ACKNOWLEDGMENTS

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