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Functional Homology of Chemotactic Methylesterases from *Bacillus subtilis* and *Escherichia coli*

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The methylesterase enzyme from *Bacillus subtilis* was compared with that from *Escherichia coli*. Both enzymes were able to demethylate methyl-accepting chemotaxis proteins (MCPs) from the other organism and were similarly affected by variations in glycerol, magnesium ion, or pH. When attractants were added to a mixture of *B. subtilis* MCPs and *E. coli* methylesterase, the rate of demethylation was enhanced. Conversely, when attractants were added to a mixture of *E. coli* MCPs and *B. subtilis* methylesterase, the rate of demethylation was diminished. These effects are what would be expected if, in these in vitro systems, the MCPs determined the rate of demethylation. These data suggest that, although the enzymes are from evolutionarily divergent organisms and are different in size, they have considerable functional homology.

Chemotaxis is the process by which motile cells migrate toward higher concentrations of attractant and away from higher concentrations of repellent. This process has been observed in evolutionarily divergent organisms (5, 13, 19). In bacteria, chemotaxis has been linked to the posttranslational modification of certain membrane proteins called methyl-accepting chemotaxis proteins (MCPs) (9, 10). These MCPs are methyl esterified on specific glutamic acid residues (1, 3, 11, 27). The number of methyl esters on an MCP changes in response to chemoeffectors (9, 10, 12, 22, 26).

Because the divergent organisms *Escherichia coli* and *Bacillus subtilis* both use this type of methylation-demethylation system, many comparisons have been made of the similarities and differences between the chemotactic processes of the two organisms. Similarities and differences in the chemoeffectors sensed have been reported (17, 18, 25), as have variations in the mechanisms of response and adaptation to similar chemoeffectors (2, 8, 9). MCPs from the two organisms have been found to have similar sites of methylation (3) as well as structural similarities (16). In addition, the chemotactic methyltransferase enzymes from the two organisms have been found to be functionally homologous (3). In this paper, we present evidence for the functional homology of the methylesterase enzymes of *E. coli* and *B. subtilis*.

MATERIALS AND METHODS

Chemicals. *S*-Adenosyl[*methyl*-^{3}H]methionine (75 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, Ill.). Electrophoresis chemicals were electrophoresis grade, and all other chemicals were reagent grade.

Bacterial strains. *B. subtilis* OI1085 (wild type for chemotaxis) and OI1100 (methyltransferase II [*cheR*] mutant) have been previously described (26). *E. coli* RP437 (wild type for chemotaxis), RP4612 (methyltransferase [*cheR*] mutant), and RP4779 (methylesterase [*cheB*] mutant) were gifts from John S. Parkinson (Department of Biology, University of Utah, Salt Lake City) and have been previously described (20).

Media. Tryptone broth contained 1% tryptone and 0.5% NaCl. Luria broth contained 1% tryptone, 0.5% NaCl, and 0.5% yeast extract. FP buffer and MT buffer were as previously described (4); ME buffer was as previously described (7); and *E. coli* FP (CFP) buffer was MT buffer plus 20% (vol/vol) glycerol and 2 mM phenylmethylsulfonyl fluoride.

Enzyme preparation. *B. subtilis* methylesterase was purified by the procedure of Goldman et al. (7). *E. coli* crude extract containing methylesterase activity was prepared by the following procedure. Frozen stock (10 μl) was thawed, placed into 1 ml of tryptone broth, and grown overnight at 37°C. This mixture was transferred to 800 ml of Luria broth and grown at 37°C with shaking to an A_{600} of about 2.5. The cells were harvested by centrifugation for 5 min (5,000 rpm in a JA14 rotor; Beckman Instruments, Inc., Fullerton, Calif.), washed twice with MT buffer, and suspended in CFP buffer to a density of 5% (wt/vol). The suspensions were passed through a French pressure cell (Aminco) three times at 18,000 lb/in^2. Cellular debris was removed by centrifugation at 17,000 × *g* for 30 min (Beckman JA20 rotor). Membranes were removed by centrifugation at 120,000 × *g* for 6 h (Beckman 70 Ti rotor). The supernatant was dialyzed for 24 h against three changes of CFP buffer and stored frozen until use in methylesterase assays. *B. subtilis* methyltransferase II was prepared as previously described (4).

Substrate preparation. *B. subtilis methyl*-^{3}H-MCPs were prepared from OI1085 membranes as previously described (7). *E. coli methyl*-^{3}H-MCPs were prepared by combining 1 ml of the membrane fraction from an enzyme preparation, after two washes with MT buffer, with 100 μl of *B. subtilis* methyltransferase and 12.5 μl of *S*-adenosyl[*methyl*-^{3}H]methionine (18 mg of membrane protein, ~0.1 mg of methyltransferase). After incubation at 22°C for 1 h, the membranes were pelleted by centrifugation at 180,000 × *g* for 3 h and washed twice with MT buffer. *methyl*-^{3}H-MCPs were stored frozen.

Enzyme assay. Methylesterase activities were assayed by the Conway cell procedure of Goldman et al. (9). The outer well contained 10 μl of *methyl*-^{3}H-MCP, enzyme, buffer, and effector in a total volume of 200 μl. After an appropriate incubation time (either 4 or 16 h), the reaction was stopped by mixing the sample with 50 μl of 2×-concentrated Laemmli sample buffer (14) by rotating the Conway cell. The cells were allowed to stand for at least 4 h to allow the

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methanol to equilibrate, and a sample was removed from the center well and assayed for [^{3}H]methanol by liquid scintillation spectroscopy.

The entire contents of the outer well were heated to 100°C for 5 min, and 25 μl was applied to a sodium dodecyl sulfate-polyacrylamide gel (10% acrylamide, 0.125% bisacrylamide) and electrophoresed as previously described (14). Fluorograms of the polyacrylamide gels were prepared by the method of Laskey and Mills (15).

RESULTS AND DISCUSSION

Demethylation by *B. subtilis* and *E. coli* methylesterases of *E. coli* and *B. subtilis* MCPs. For assessment of the activity of *E. coli* methylesterase toward *B. subtilis* MCPs, crude extract from *E. coli* was mixed with *methyl*-^{3}H-MCPs from *B. subtilis* in a Conway cell, assayed for demethylation, and found to cause methanol formation (Fig. 1A). Conversely, for assessment of the activity of *B. subtilis* methylesterase toward *E. coli* MCPs, the purified methylesterase from *B. subtilis* was mixed with *methyl*-^{3}H-MCPs from *E. coli* in a Conway cell, assayed for demethylation, and found to cause methanol formation (Fig. 1B). The extent of the reaction and the time course of methanol formation were similar to those obtained from the demethylation of *methyl*-^{3}H-MCPs by enzyme from the same organism (data not shown). The demethylation of *B. subtilis methyl*-^{3}H-MCPs by *E. coli* crude extract was the result of the presence of the methylesterase enzyme, since an extract from a methylesterase-defective mutant of *E. coli* was unable to demethylate *B. subtilis methyl*-^{3}H-MCPs (data not shown).

Effect of glycerol and magnesium ion on *B. subtilis* and *E. coli* methylesterase activities. Glycerol has been shown to both stabilize and activate the methylesterase activity of *B. subtilis* in vitro (7). A time course of the demethylation of *B. subtilis methyl*-^{3}H-MCPs by *E. coli* crude methylesterase was determined in the presence or absence of 20% (vol/vol) glycerol. Glycerol activated the *E. coli* methylesterase activity about 10-fold (Fig. 2). That this was an activation and not a protection from denaturation was evidenced by the stability of the enzyme activity in the absence of glycerol. No inactivation of the *E. coli* methylesterase activity in the absence of glycerol was observed. In addition, the activities of methylesterase enzymes from *E. coli* and *B. subtilis* toward *methyl*-^{3}H-MCPs from *E. coli* were found to be stimulated by the presence of glycerol (data not shown).

Mg^{2+} has been demonstrated to be a requirement for the demethylation of *B. subtilis* membranes by *B. subtilis* methylesterase (7). A Conway cell assay for the ability of Mg^{2+} to stimulate the *E. coli* crude methylesterase to demethylate *B. subtilis methyl*-^{3}H-MCPs was performed. In addition, methylesterases from *B. subtilis* and *E. coli* were assayed for their requirement for Mg^{2+} when demethylating *E. coli methyl*-^{3}H-MCPs. We found that 1 mM Mg^{2+} was required for the demethylation of *B. subtilis methyl*-^{3}H-MCPs, while *E. coli methyl*-^{3}H-MCPs were demethylated in the presence of only 0.01 mM Mg^{2+} (Table 1). Thus, the requirement for Mg^{2+} was a membrane property and not an enzyme property, since the membrane type determined whether Mg^{2+} was required.

Effect of pH on methylesterase activities. The ability of *B. subtilis* methylesterase to demethylate *E. coli* or *B. subtilis* MCPs at various pHs was determined. In addition, the ability of *E. coli* crude methylesterase to demethylate *B. subtilis* MCPs at various pHs was assayed. The pH-versus-activity profiles of the two methylesterase activities were very similar (data not shown). Both enzymes had very little activity below pH 6 and maximal activity at pH 7 and higher. The activity profile of *B. subtilis* methylesterase demethylating *E. coli* MCPs in the presence of various pHs was indistinguishable from the activity profile of *B. subtilis* methylesterase demethylating *B. subtilis* MCPs.

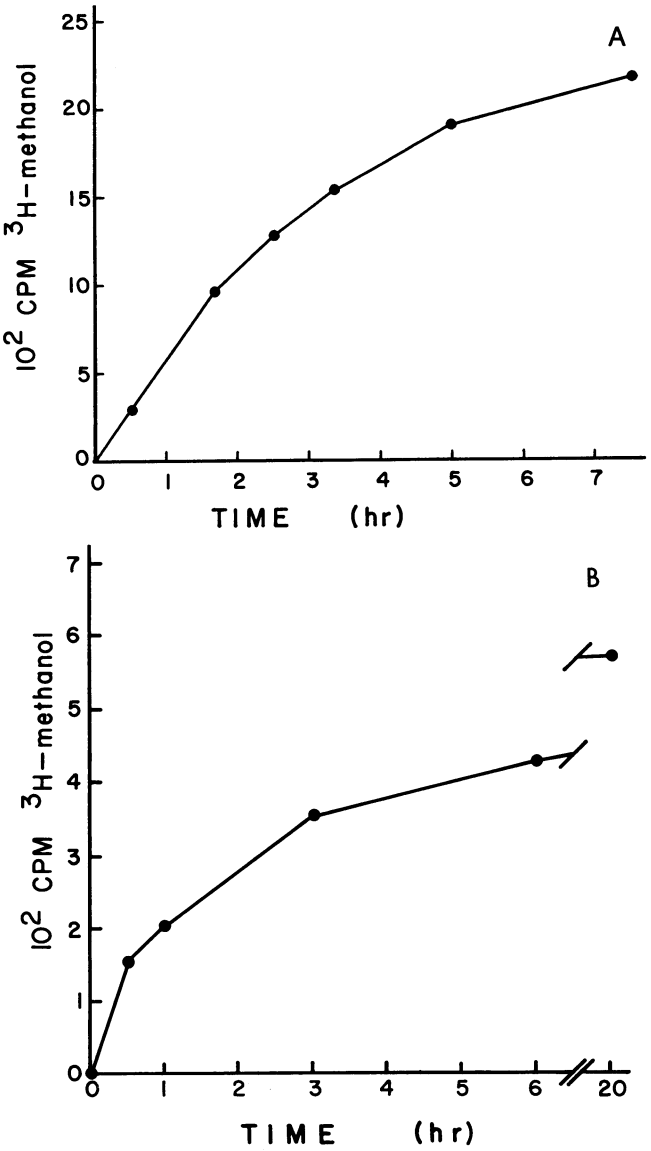

FIG. 1. Time course of methanol formation from MCPs catalyzed by heterologous methylesterases. See Materials and Methods for experimental procedures. (A) *E. coli* MCPs and *B. subtilis* methylesterase. (B) *B. subtilis* MCPs and *E. coli* methylesterase.

Stimulation and inhibition of methylesterase activities by attractants and repellents. Aspartate is an attractant of both *E. coli* and *B. subtilis*. However, aspartate inhibits the demethylation of *E. coli* MCPs in vivo and stimulates a cycle(s) of demethylation and methylation of *B. subtilis* MCPs. *E. coli* crude methylesterase was mixed with *B. subtilis methyl*-^{3}H-MCPs, and the effect of the addition of aspartate on the rate of methanol formation was observed. Aspartate stimulated the demethylation of *B. subtilis methyl*-^{3}H-MCPs by *E. coli* methylesterase (Table 2). Furthermore, the change in demethylation rate with various amounts of

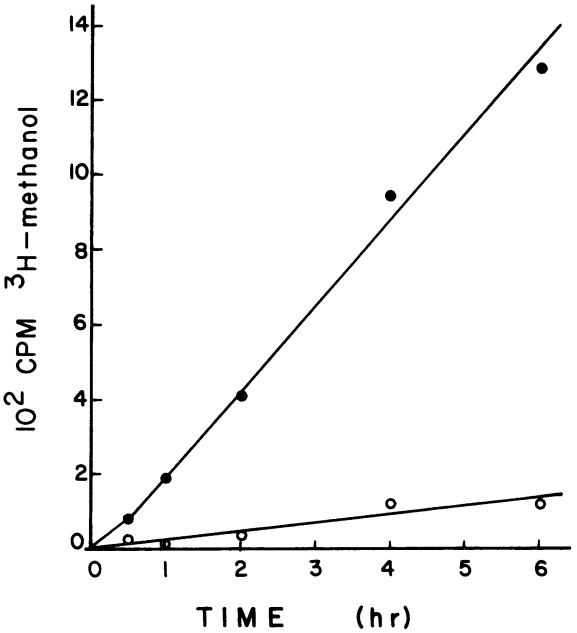

FIG. 2. Effect of glycerol on the activity of *E. coli* methylesterase. *E. coli* crude supernatant, prepared as described in Materials and Methods, was mixed with ^{3}H-labeled *B. subtilis* wild-type membranes in Conway cells in the presence (●) or absence (○) of 20% glycerol.

aspartate showed a concentration dependence similar to that expected from an active species with a K_d of about 10 mM, which is the behavioral "K_d" of *B. subtilis* for aspartate (18).

When aspartate and serine were added to a mixture of methylesterase from *B. subtilis* and *methyl*-^{3}H-MCPs from *E. coli*, the rate of demethylation of the *E. coli* MCPs was diminished, as indicated by the decreased radioactivity found in the center well of a Conway cell in an assay for methylesterase activity and as compared with the control samples, which contained no serine or aspartate. Conversely, when a combination of repellents was added to the same mixture, the rate of demethylation of the *E. coli* MCPs was enhanced, as indicated by the increased radioactivity found in the center well of a Conway cell (Table 2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography of samples from the outer wells of these Conway cells revealed less demethylation in the sample mixtures which contained aspartate and serine (Fig. 3). Thus, when attractants or repellents were added to a mixture of *B. subtilis* methylesterase and *E. coli methyl*-^{3}H-MCPs, the effect on the rate of demethylation was that which would have been expected if the *E. coli* MCPs were a locus of regulation by an attractant or a repellent.

TABLE 1. Effect of magnesium ion concentration on MCP demethylation

methyl-^{3}H-MCP source	Methylesterase source	Demethylation (cpm)[a] with Mg^{2+} at:	
		10^{-5} M	10^{-3} M
E. coli	*E. coli*	2,006	1,504
E. coli	*B. subtilis*	3,012	2,764
B. subtilis	*E. coli*	305	2,261

[a] [^{3}H]Methanol counts per minute above the background in Conway cell assays.

TABLE 2. Effect of attractants and repellents on MCP demethylation

MCP and enzyme	Addition[a]	Demethylation[b]
E. coli MCPs and *B. subtilis* methylesterase	Buffer	100 ± 2.3
	70 μM Aspartate[c]	88 ± 1.0
	70 μM Aspartate + 3 mM serine	75 ± 2.4
	Repellent mixture[d]	121 ± 3 (estimated)
B. subtilis MCPs and *E. coli* methylesterase	Buffer	100 ± 2.9
	10 mM Aspartate[e]	142 ± 3.5
	100 mM Aspartate	180 ± 8.4

[a] Addition to the Conway cell assay mixture described in Materials and Methods.

[b] Percent of buffer control value ± standard error of the mean.

[c] Ten times the *E. coli* K_d for this attractant (6).

[d] The mixture contained indole (0.3 mM), acetate (20 mM), and leucine (20 mM) (pH 7); this is a standard repellent mixture for *E. coli* chemotaxis assays (21).

[e] Behavioral K_d for this attractant with *B. subtilis*.

In summary, the demethylation of *B. subtilis* MCPs is stimulated by the addition of attractants, while the demethylation of *E. coli* MCPs is retarded by attractants and stimulated by repellents (21, 23). The methylesterase enzymes are not directly controlled by chemoeffectors, since

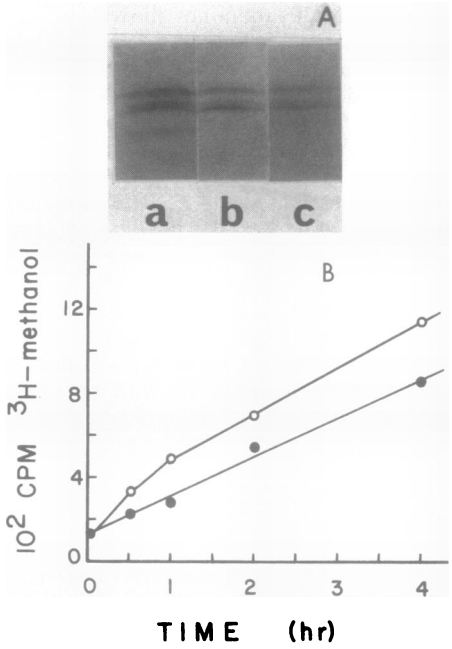

FIG. 3. (A) Fluorograph of aspartate- and serine-mediated inhibition of *E. coli* MCP demethylation by *B. subtilis* methylesterase. ^{3}H-labeled *E. coli* membranes were mixed with *B. subtilis* methylesterase in the presence or absence of 70 μM aspartate and 3 mM serine. Demethylation was stopped after 20 h, and samples were prepared and fluorographed as described in Materials and Methods. Lanes: a, zero-time control; b, aspartate and serine; c, no addition. (B) Effect of aspartate and serine on methanol formation caused by demethylation of *E. coli* MCPs. ^{3}H-labeled *E. coli* membranes were mixed with *B. subtilis* methylesterase in the presence (●) or absence (○) of 70 μM aspartate and 3 mM serine. Conway cell assay reactions were stopped at the indicated times by mixing with sodium dodecyl sulfate sample buffer.

the change in the methylesterase activity of a heterologous system is MCP specific. Therefore, the MCP (substrate) source, not the esterase source, determines the change in the rate of demethylation. Since the MCP determines the change in the methylesterase activity in response to a chemoeffector, the chemoeffector must directly affect MCP conformation in such a way as to render the methyl esters more susceptible to hydrolysis.

The activity of *E. coli* methylesterase in vivo is also subject to a global control in which the addition of MCP I attractant inhibits not only the demethylation of MCP I but also the demethylation of MCP II (24). Since the addition of MCP II attractant in vitro does not reduce methanol production to control (no enzyme) levels, this global control mechanism must involve other factors not present in sufficient concentrations in this assay system.

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