JOURNAL OF BACTERIOLOGY, Mar. 1988, p. 1393–1395
0021-9193/88/031393-03$02.00/0

Vol. 170, No. 3

Methanol Production by *Mycobacterium smegmatis*

LOIS S. WEISMAN AND CLINTON E. BALLOU*

Department of Biochemistry, University of California, Berkeley, California 94720

Received 21 September 1987/Accepted 24 November 1987

***Mycobacterium smegmatis* cells produce [^{3}H]methanol when incubated with [*methyl*-^{3}H]methionine. The methanol is derived from *S*-adenosylmethionine rather than methyltetrahydrofolate. *M. smegmatis* cells carboxymethylate several proteins, and some of the methanol probably results from their demethylation, but most of the methanol may come from an unidentified component with a high gel mobility. Although methanol in the medium reached 19 μM, it was not incorporated into the methylated mannose polysaccharide, a lipid carrier in this organism.**

Mycobacterium smegmatis cells synthesize a 3-*O*-methylmannose-containing polysaccharide (MMP) (7) that contains two types of methyl groups, the ethers on position 3 of the mannoses and the acetal of the methyl aglycon at the reducing end of the polymer chain. Cell extracts have a 3-*O*-methyltransferase activity (13) that is involved in synthesis of 3-*O*-methylmannose, but the origin of the methyl aglycon is unclear (14), although both types of methyl groups become labeled when *M. smegmatis* cells are grown in the presence of [*methyl*-^{3}H]methionine (7). We report here that *M. smegmatis* cells release [^{3}H]methanol into the medium when grown in the presence of [*methyl*-^{3}H]methionine, but this free methanol is not utilized in MMP biosynthesis. We believe that this is the first report of methanol production from methionine by a nonmotile bacterium.

Paper chromatography was performed on no. 1 filter paper (Whatman, Inc., Clifton, N.J.), and sugars were visualized with alkaline silver nitrate (1). Radioactivity was determined by cutting the chromatogram into 1-cm strips, which were wetted with water and counted in 10 ml of Biofluor (New England Nuclear Corp., Boston, Mass.). Gas chromatography of short-chain alcohols was performed on a model 920 chromatograph (Varian) equipped with a thermal conductivity detector coupled to a gas flow radioactivity monitor (Nuclear-Chicago Corp., Des Plaines, Ill.). A glass column (0.25 in. by 10 ft [ca. 0.64 by 300 cm]) packed with Porapak Q (80/100 mesh) was used at 150°C, with a helium flow rate of 30 ml/min. Propane was passed through the radioactivity monitor at 60 ml/min.

For most experiments, cells were grown on a Casamino Acids medium (5), but for labeling cells with [*methyl*-^{3}H]methionine, methionine assay medium (Difco Laboratories, Detroit, Mich.) was substituted for the Casamino Acids. Protein extracts were electrophoresed (6) on a 12.5% sodium dodecyl sulfate-polyacrylamide gel with a 5% stacking gel, and the gel was fixed in methanol-acetic acid and stained with Coomassie blue. After being destained with acetic acid, the gel was dried and cut into 2-mm slices. Each slice was incubated in 100 μl of 1 M Na_2CO_3 (pH 11) for 30 min at 23°C, after which 0.5 ml of methanol was added, collected by distillation at 85°C, and counted (11).

Production in *M. smegmatis* cells of [^{3}H]methanol from [^{3}H]methionine. Cells grown in a medium (10) containing 50 μM methionine as the sole amino acid with [*methyl*-^{3}H]methionine (30 μCi) and [^{35}S]methionine (1 μCi) showed an apparent differential uptake of the two isotopes (Fig. 1). After 52 h of growth, the cell filtrate contained both ^{3}H and ^{35}S, but only ^{3}H was present in the distillate. Analysis of the distillate by gas chromatography showed that the predominant labeled compound was methanol, although some labeled water was also present (Fig. 2). When cells were grown on a Casamino Acids medium, the filtrate at stationary phase contained up to 19 μM methanol. Methanol production from a culture containing both L-[*methyl*-^{14}C]methionine and L-[*methyl*-^{3}H]methionine showed that the ^{3}H/^{14}C ratio remained unchanged, as expected if methanol was formed by means of *S*-adenosylmethionine rather than methyltetrahydrofolate.

***M. smegmatis* production of saponifiable methylated proteins.** Methanol is formed in chemotactic bacteria from reversible methylation of proteins (12). *M. smegmatis* cells were incubated for 5 or 60 min in 1 ml of methionine-free medium containing 200 μCi of L-[*methyl*-^{3}H]methionine (80 Ci/mmol), and the total protein from a portion of each culture was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Two protein bands from the 5-min pulse released [^{3}H]methanol upon alkaline treatment, whereas the 60-min pulse shows methanol released from five protein bands (Fig. 3). The majority of the methanol, however, came from material at the gel front that was fixed with 50% methanol and 10% acetic acid. Because aspartyl and isoaspartyl methyl esters are unstable during gel electrophoresis, our result provides a minimal estimate of the total protein methyl ester in the cell extract.

Mycobacterial cell extract synthesis of ethyl D-mannoside but not methyl D-mannoside. A cell extract (7 ml) was incubated at 37°C for 45 min with 0.24 mM ethanol and 50 μM GDP-mannose containing 15 μCi of GDP-[^{3}H]mannose. Unreacted GDP-mannose and charged products were removed with Dowex-1 and Dowex-50, and 1.5 μCi of tritium was recovered in neutral products, which comigrated with ethyl α-D-mannoside (Fig. 4A) when chromatographed on no. 1 filter paper (Whatman) in ethyl acetate-propanol-water (5:3:2 vol/vol/vol). Acid hydrolysis of the product yielded the expected [^{3}H]mannose (Fig. 4B). $NaBH_4$ reduction of the product followed by acid hydrolysis also gave [^{3}H]mannose, which confirms the absence of a free reducing end in the labeled product.

Because *M. smegmatis* cells produce methanol, it seemed possible that they also make methyl α-D-mannoside to initiate biosynthesis of MMP. However, methylmannoside was not detected after incubations of cell extracts with

* Corresponding author.

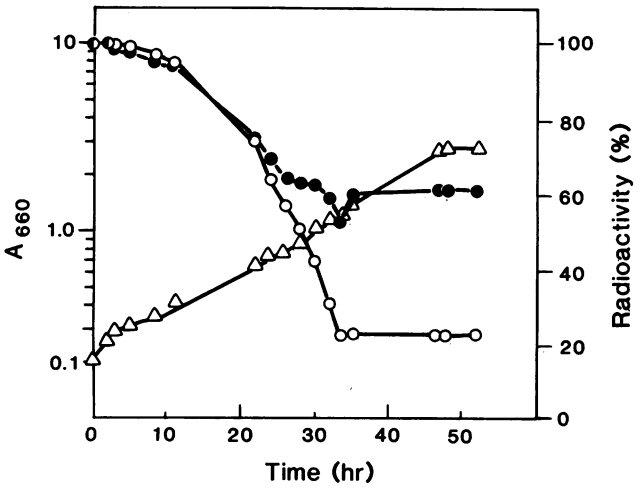

FIG. 1. A differential uptake of isotope is observed when *M. smegmatis* cells are grown in the presence of [*methyl*-^{3}H]methionine and [^{35}S]methionine. Optical density at 660 nm (cell mass) (△) and ^{35}S (○) and ^{3}H (●) remaining in the culture filtrate are plotted as a function of time.

GDP-mannose and methanol or with mannose and methanol, and labeled methyl α-D-mannoside could not be isolated from cells grown on [^{3}H]mannose or [*methyl*-^{3}H]methionine.

[^{3}H]methanol not incorporated into MMP. Cells grown with [*methyl*-^{3}H]methionine produce MMP which has labeled methyl ethers on the mannoses and a labeled methyl aglycon (7). Although the methyl aglycon could come directly from *S*-adenosylmethionine, the demonstration that *M. smegmatis* cells produce large amounts of methanol suggested that the alcohol might be a mannose acceptor for MMP synthesis. *M. smegmatis* cells were incubated in 10 ml of Casamino Acids medium containing 5 mCi of [^{3}H]methanol and 10 mM methanol. After 15 min, the culture was used to inoculate a 90-ml culture which was grown to stationary phase (24 h at 23°C). Approximately 100 nmol of MMP was isolated from these cells (5). If each MMP

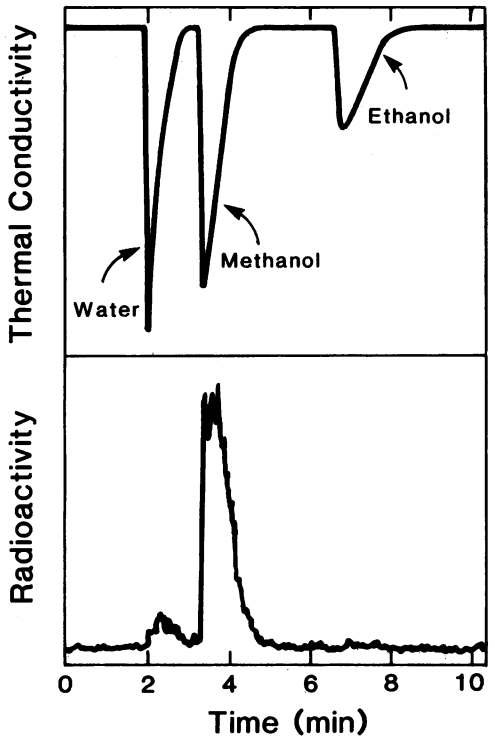

FIG. 2. Identification of [^{3}H]methanol in the *M. smegmatis* culture filtrate by gas chromatography of the distillate from the culture filtrate from *M. smegmatis* cells grown in the presence of [*methyl*-^{3}H]methionine and [^{35}S]methionine (Fig. 1). The top line shows the change in thermal conductivity (unlabeled reference material), and the bottom trace shows changes in radioactivity from the culture filtrate. Most of the ^{3}H label comigrated with methanol.

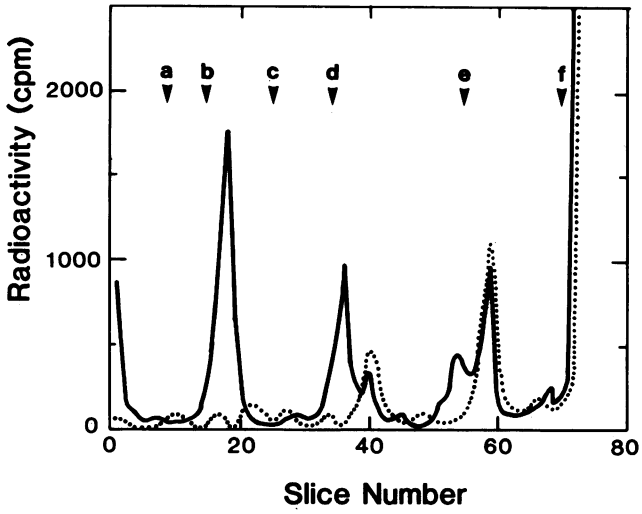

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of alkali-labile methyl esters. Extracts from *M. smegmatis* cultures were labeled for 5 min (· · · ·) or 60 min (——) and analyzed for alkali-labile radioactivity. The arrows indicate the positions of protein standards of 94 (a), 68 (b), 45 (c), 30 (d), 20 (e), and 14.4 (f) kilodaltons.

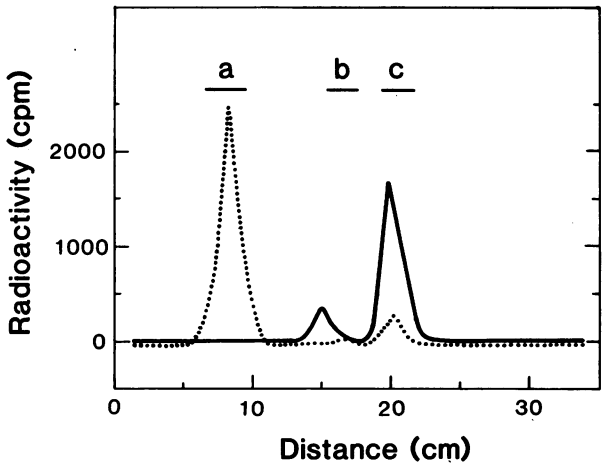

FIG. 4. Paper chromatographic characterization of ethyl α-D-mannoside. Cell extracts, incubated with GDP-[^{3}H]mannose in the presence of ethanol, were treated with Dowex-1 and Dowex-50, and the neutral product was chromatographed on a Bio-Gel P-2 (−400-mesh) column run in water and then on Whatman no. 1 paper irrigated with ethyl acetate-propanol-water (——). The same material was hydrolyzed in acid and rechromatographed (· · · ·). The bars indicate the positions of standard mannose (a), methyl α-D-mannoside (b), and ethyl α-D-mannoside (c).

molecule had incorporated one molecule of methanol, the polymer would have contained 5×10^6 cpm. The MMP was hydrolyzed in 1 N trifluoroacetic acid at 120°C for 90 min, which would release a methyl aglycon as methanol, but none of the radioactivity in the hydrolysate was volatile.

We have demonstrated that *M. smegmatis* cells release [^{3}H]methanol into the medium when grown with [*methyl*-^{3}H]methionine. Because this occurs even in the presence of a full complement of amino acids, it is unlikely that the methanol is a product of methionine catabolism; moreover, *S*-adenosylmethionine and not methyltetrahydrofolate is the apparent intermediate in methanol formation. We were intrigued by the observation that ethyl α-D-mannoside is produced when GDP-mannose and ethanol are incubated in a cell extract, but we obtained no indication that methyl α-D-mannoside is similarly formed, so this compound may not be involved in initiation of MMP biosynthesis. Thus, the activity that forms ethylmannoside may be an α1→6-mannosyltransferase that can utilize ethanol but not methanol as an acceptor.

Enteric bacteria produce methanol from demethylation of a membrane protein that regulates chemotaxis (12). Reversible methylation of proteins is ubiquitous among eucaryotes (9) and may be involved in the repair of D-aspartyl and L-isoaspartyl residues (2), a type of reversible methylation also observed in *Salmonella* species (8). Methanol produced by the nonmotile *M. smegmatis* cells probably also comes from reversible protein methylation, and the most highly labeled protein had the same molecular weight (60,000) as the methylated chemotaxis protein (3). Most of the alkali-labile methanol in cell extracts, however, comes from a small protein or lipidlike material that is not extracted during fixation (4).

We thank Daniel Chelsky for performing the sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the mycobacterial extracts for alkali-labile methanol.

This work was supported by U.S. Public Health Service grant AI-12522 and National Science Foundation grant PCM 80-23388. L.S.W. was supported by U.S. Public Health Service training grant GM-07232.

LITERATURE CITED

1. **Anet, E. F. L. J., and T. M. Reynolds.** 1954. Isolation of mucic acid from fruits. Nature (London) **174**:930.
2. **Clarke, S.** 1985. Protein carboxyl methyltransferases: two distinct classes of enzymes. Annu. Rev. Biochem. **54**:479–506.
3. **DeFranco, A. L., and D. E. Koshland, Jr.** 1980. Multiple methylation in processing of sensory signals during bacterial chemotaxis. Proc. Natl. Acad. Sci. USA **77**:2429–2433.
4. **Dzandu, J. K., and R. M. Johnson.** 1980. Membrane protein phosphorylation in intact normal and sickle cell erythrocytes. J. Biol. Chem. **255**:6382–6386.
5. **Gray, G. R., and C. E. Ballou.** 1975. Methylated polysaccharide activators of fatty acid synthase from *Mycobacterium phlei*. Methods Enzymol. **35B**:90–95.
6. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
7. **Maitra, S. K., and C. E. Ballou.** 1977. Heterogeneity and refined structures of 3-*O*-methyl-D-mannose polysaccharides from *Mycobacterium smegmatis*. J. Biol. Chem. **252**:2459–2469.
8. **O'Connor, C. M., and S. Clarke.** 1985. Specific recognition of altered polypeptides by widely distributed methyltransferases. Biochem. Biophys. Res. Commun. **132**:1144–1150.
9. **Paik, W. K., and S. Kim.** 1980. Methylation of free carboxyl groups of proteins, p. 202–231. *In* A. Meister (ed.), Protein methylation. John Wiley & Sons, Inc., New York.
10. **Prasad, R., V. Kalra, and A. Brodie.** 1976. Different mechanisms of energy coupling for transport of various amino acids in cells. J. Biol. Chem. **251**:2493–2498.
11. **Stock, J. B., and D. E. Koshland, Jr.** 1981. Changing reactivity of receptor carboxyl groups during bacterial sensing. J. Biol. Chem. **256**:10826–10833.
12. **Toews, M. L., and J. Adler.** 1979. Methanol formation *in vivo* from methylated chemotaxis proteins in *Escherichia coli*. J. Biol. Chem. **254**:1761–1764.
13. **Weisman, L. S., and C. E. Ballou.** 1984. Biosynthesis of the mycobacterial methylmannose polysaccharide. Identification of a 3-*O*-methyltransferase. J. Biol. Chem. **259**:3464–3469.
14. **Yamada, H., R. E. Cohen, and C. E. Ballou.** 1979. Characterization of 3-*O*-methyl-D-mannose polysaccharide precursors in *Mycobacterium smegmatis*. J. Biol. Chem. **254**:1972–1979.