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NOTES

Chloramphenicol Acetyltransferase Should Not Provide Methanogens with Resistance to Chloramphenicol

GREGORY S. BECKLER, LEONARD A. HOOK, AND JOHN N. REEVE*
Department of Microbiology, The Ohio State University, Columbus, Ohio 43210

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Growth of the four methanogens investigated was inhibited by chloramphenicol-3-acetate; therefore, introduction of chloramphenicol acetyltransferase-encoding genes should not confer chloramphenicol resistance on these methanogens. Reduction of the aryl nitro group of chloramphenicol produced a compound which did not inhibit the growth of these methanogens.

We recently began a research program to develop gene transfer systems for members of the methanogenic group of archaebacteria (7; P. T. Hamilton and J. N. Reeve, *in* W. R. Strohl and O. H. Tuovinen, ed., *Microbial Chemoautotrophy*, in press; L. A. Hook, R. E. Corder, P. T. Hamilton, J. I. Frea, and J. N. Reeve, *in* W. R. Strohl and O. H. Tuovinen, ed., *Microbial Chemoautotrophy*, in press). Our initial goals were to identify compounds inhibitory to the growth of methanogens and subsequently to isolate mutant strains of methanogens whose growth was resistant to these compounds. Antibiotic resistance could then be used as a selective agent in development of genetic exchange systems. We confirmed previous results that the growth of all methanogenic species tested is inhibited by monensin, bromoethanesulfonate, and chloramphenicol (CAM) and added to this list the compounds leucinostatin, metronidazole, and pyrrolnitrin (2, 4; Hook et al., in press). The most attractive candidate compound from this list for use as a selective agent would appear to be CAM. DNA sequences encoding chloramphenicol acetyltransferase (CAT), which confer CAM resistance to many bacterial species, are readily available as components of several well-characterized plasmids and transposons. We argued, therefore, that if CAT activity detoxified CAM with respect to the growth of methanogens, it would be appropriate to attempt to introduce known CAT genes into methanogens by using CAM resistance to select for transformants. In view of the fact that Elhardt and Böck (2) had already shown that CAM does not inhibit ribosome function in methanogens, we decided to determine first whether acetylation of CAM would prevent it from inhibiting the growth of methanogens before attempting to introduce CAT genes into methanogenic species.

CAM was acetylated in vitro with CAT (EC 2.3.1.28) from *Escherichia coli* (Sigma Chemical Co., St. Louis, Mo.). Acetylation of CAM did not reduce the growth-inhibiting activity of the compound for *Methanococcus voltae* but did detoxify CAM with respect to the growth of *E. coli* (Fig. 1A and B). In experiments of the same type as shown in Fig. 1 for *M. voltae*, chloramphenicol-3-acetate was also found to inhibit the growth of *Methanococcus vannielii*, *Methanococcus deltae*, and *Methanobrevibacter smithii*. It therefore appears that CAT activity would not confer CAM resistance to methanogens, and introduction of CAT genes into methanogens would not provide a useful selectable genetic trait. It was previously reported that CAM may interact with dehydrogenases in methanogens (5) and that the aryl nitro group of CAM can act as an oxidizing agent under anaerobic conditions (6). It seemed probable, therefore, that the ability of CAM to inhibit the growth of methanogens resides in the oxidizing activity of the aryl nitro group of CAM. Titanous chloride was used to reduce this nitro group to an amine (3), and the reduced compound [1-(*p*-aminophenyl)-2-dichloroacetamido-1,3-propanediol] did not inhibit the growth of *M. voltae* nor, as previously reported (9), the growth of *E. coli* (Fig. 1C and D). The conversion of CAM to its reduction product [1-(*p*-aminophenyl)-2-dichloroacetamido-1,3-propanediol] was confirmed by analysis of the reaction products by thin-layer paper chromatography and detection of nitro and aryl groups as described previously by Glazko et al. (3) and Smith and Worrel (8).

The results of this study show that whereas CAM is a very effective inhibitor of methanogen growth, CAT activity is not of value in creating CAM resistance in methanogens. Exposure of metronidazole and pyrrolnitrin to the reducing conditions produced by titanous chloride also converted both of these compounds to compounds which no longer inhibited the growth of methanogens (results not shown), indicating that these compounds may also inhibit the growth of methanogens by acting as oxidizing agents.

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* Corresponding author.

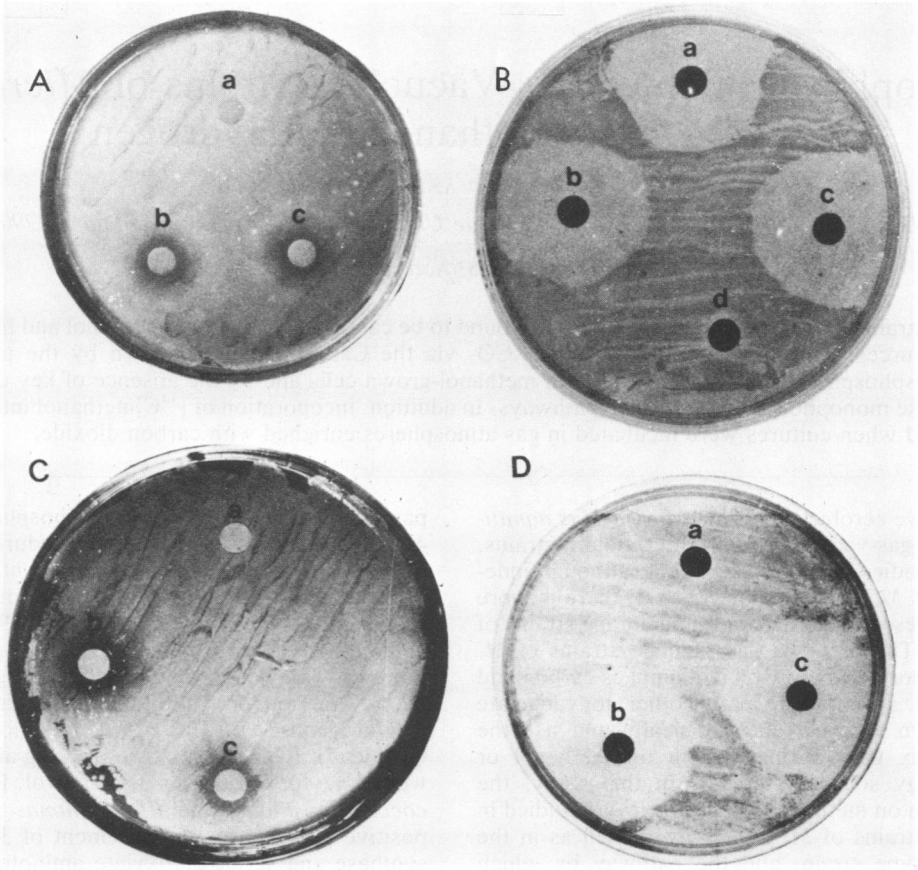

FIG. 1. Sensitivity of *M. voltae* and *E. coli* to CAM and derivatives of CAM. CAM was converted to chloramphenicol-3-acetate by incubation with CAT for 14 h at 37°C. The reaction mixture (600 μl) contained the following compounds dissolved in a solution buffered to pH 7.8 with 10 mM Tris-hydrochloride–5% (vol/vol) ethanol: 0.3 mg of CAM, 0.9 mg of acetyl coenzyme A, and 75 U of CAT. Control reactions were placed at 100°C for 5 min immediately after the reagents were mixed or were constructed with the omission of CAM. Portions (20 μl, equivalent to 10 μg of CAM) of the reaction mixtures were spotted on sterile filter paper disks which were dried, placed in a vacuum for 1 h to degas, and then placed on freshly seeded lawns of *M. voltae* (on MMM plates containing 3% [wt/vol] NaCl, 20 mM $MgCl_2$, 0.2% [wt/vol] Casamino Acids [Hook et al., in press]) or *E. coli* C600 (on LB plates [1]). Plates inoculated with *M. voltae* were incubated anaerobically at 37°C as previously described (Hook et al., in press). Plates inoculated with *E. coli* were incubated aerobically at 37°C. (A) *E. coli* exposed to complete reaction (a), heat-inactivated reaction (b), 10 μg of CAM (c). (B) *M. voltae* exposed to complete reaction (a), heat-inactivated reaction (b), 10 μg of CAM (c), or reaction mixture minus CAM (d). CAM was reducted by incubation with titanous chloride (3) under anaerobic conditions. $TiCl_3$ (5.6 ml; 20% [wt/vol]) was mixed with 37.5 ml of concentrated HCl. The mixture was boiled under an atmosphere of CO_2, diluted by the addition of 450 ml of degassed water, sparged with H_2, placed in a sealed 1-liter Wheaton bottle containing 10 lb/in^2 of H_2, and autoclaved. CAM solution (250 μl; 2 mg of CAM in 200 μl of ethanol plus 1.8 ml of water) was mixed with 2 ml of $TiCl_3$ solution plus 4 ml of anaerobic water. Control reactions replaced the $TiCl_3$ solution with 2 ml of anaerobic water. Two milliliters of 1 N NaOH was added to precipitate titanium oxide, which was then removed by centrifugation (7 min at 2,000 rpm; Sorvall SS-1 rotor). The supernatant was filtered through a 0.45-μm (pore size) filter (Millipore Corp., Bedford, Mass.), adjusted to pH 7 by the addition of HCl, and dried in a SpeedVac concentrator (Savant Instruments, Inc., Hicksville, N.Y.), and the resulting reduction product of CAM was redissolved in 500 μl of water. Filter paper disks were impregnated with portions of this solution (20 μl, equivalent to 10 μg of CAM), dried, degassed, and placed on lawns of *E. coli* C600 or *M. voltae* as described above. (C) *E. coli* exposed to complete reaction (a), reaction minus $TiCl_3$ (b), or 10 μg of CAM (c). (D) *M. voltae* exposed to complete reaction (a), reaction minus $TiCl_3$ (b), or 10 μg of CAM (c). Plates seeded with *E. coli* were photographed without staining against a dark background. Plates seeded with *M. voltae* were stained (1 min of exposure to 95% [vol/vol] ethanol; 3 min of exposure to an aqueous solution of safranin [0.25% {wt/vol}; MCB Reagents, Cincinnati, Ohio]), and washed with water immediately before their removal from the anaerobic environment and before being photographed against a light-colored background.

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