Inhibition of *Bacillus megaterium* by a Trimethylamine Oxide-Associated Browning Reaction Product

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Heated combinations of trimethylamine oxide (TMAO) and culture media (tryptone, glucose, yeast extract broth or a defined minimal medium), or heated TMAO and glucose, contained substance(s) that inhibited growth of *Bacillus megaterium*. Inhibition was expressed primarily as an increase of the lag phase of growth; the logarithmic growth rate was comparable to control cultures. The addition of unheated TMAO to the culture media had no effect on growth. Results suggested that TMAO was decomposed during heating and that dimethylamine, one of the degradation products, reacted with glucose by a Maillard-Amadori reaction to produce the inhibitory substance(s).

Trimethylamine oxide (TMAO) occurs in most marine animals (2) and is of interest because of the possibility that it influences the growth of organisms in seafoods. Generally, seafoods are more susceptible to spoilage by gram-negative bacteria than by gram-positive bacteria; it has been observed that incorporation of TMAO in a plating medium inhibited microbial growth, particularly growth of gram-positive organisms (1). However, TMAO was added before the medium was autoclaved, introducing the possibility that inhibition was the result of a heat-induced reaction and not attributable to the naturally occurring compound. Therefore, studies were conducted on the influence of heat on TMAO-associated inhibition.

**MATERIALS AND METHODS**

Preparation of inoculum and assay of growth in test media. *Bacillus megaterium* QM 1551 (5) was maintained on slants of plate count agar (Difco). The slants were incubated at 32 C for 24 hr and stored at 4 C until needed. Prior to each experiment, the organism was transferred to the basal experimental medium and incubated at 32 C for 16 hr on a reciprocal shaker. After incubation, cells were enumerated by direct microscopic count and appropriately diluted for inoculation into optically matched test tubes (150 by 18 mm) containing 10 ml of the test medium. These cultures were incubated at 32 C on a reciprocal shaker, and periodically the growth was measured spectrophotometrically at 600 nm (Spectronic-20 colorimeter, Bausch & Lomb Inc.). The inoculum used in these experiments consisted of vegetative cells. Spores were rarely observed in the above 16 hr cultures, and any which may have been present were effectively diluted out before inoculation of the test media.

Test media and chemicals. Broth (TGY) was prepared containing tryptone, 0.5% (Difco); yeast extract, 0.25% (Difco); and glucose, 0.01%. The defined medium of Levinson and Hyatt (5) was prepared from filter-sterilized stock solutions of glucose, the sodium salt of  L-glutamic acid, and mineral salts. The glucose and sodium glutamate solutions were 25 times the concentration of the final medium, and the mineral salts stock solution contained a fivefold concentration of these ingredients. The medium was prepared in 10-ml quantities with 2 ml of the mineral salts solution, 0.4 ml of both the glucose and sodium glutamate stock solutions, and 7.2 ml of water. To study the effect of heating TMAO with various components of defined medium, the following procedure was used. Solutions of TMAO and glucose, TMAO and sodium glutamate, and glucose, all at a concentration of 0.05 M, were heated in 0.01 M sodium phosphate buffer (pH 7.0). Then, 1 ml of each solution was added to separate tubes containing the above required amounts of minerals, glucose, and sodium glutamate plus 6.2 ml of water. A medium containing TMAO was prepared by mixing 5 ml of 0.26 M TMAO, heated in the above buffer with 5 ml of unheated, double-strength defined medium. Analytical grade glucose and mineral salts were obtained from the Fisher Scientific Co., Atlanta, Ga., and sodium glutamate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Media and all test solutions were sterilized by autoclaving at 121 C for 15 min ("heated") or by filtration ("unheated") through a 0.45 μm filter (Millipore Corp., Bedford, Mass.).

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TMAO was synthesized by the method of Meisenheimer and Bratring (7) from technical grade 25% aqueous trimethylamine (Matheson, Coleman and Bell, East Rutherford, N.J.) and 30% hydrogen peroxide (Allied Chemicals, Morristown, N.J.). The TMAO was crystallized 2X from a 1:1 mixture of reagent grade ethanol and ether. The uncorrected melting point of the crystals was 96°C, which agrees with the published value (7). Studies of TMAO inhibition were begun with this material, and, for consistency, the material was used throughout the study, although preliminary trials showed the test organism responded similarly to TMAO from commercial sources. Dimethylamine (DMA; 25% in water) was obtained from Matheson, Coleman and Bell.

Ultraviolet (UV) spectroscopy. The UV spectrum was measured with a double beam recording DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) in a 1-cm silica cell.

Gas chromatography. Gas chromatographic analysis was used to identify volatile amines in heated defined medium containing 1% TMAO. A 10-ml sample was neutralized with 1 N HCl, freeze-dried, and redissolved in 1 ml of alkaline 95% ethanol. A 1-µliter sample was injected into a stainless steel column (180 cm by 8 mm) containing 5% carbowax-20M on fire 1% TMAO than in heated medium with 95% TMAO. Filter-sterilized TMAO (1%) in unbeated broth stimulated growth. Heating TGY had an adverse effect on the growth of B. megaterium; the lag phase in the heated control was approximately 2 hr longer than in the unheated control.

Experiments in defined medium indicated that the inhibitor was formed by a reaction between TMAO and glucose (Table 1). Absorbance measurements taken at periodic intervals showed that the heated TMAO-glucose solution inhibited growth of the approximate 100 cells/ml inoculum for the 48-hr test period. The addition of heated TMAO alone to the medium had little effect on growth, although the concentration was approximately 26 times the TMAO concentration in the medium containing the TMAO-glucose solution. The addition of heated glucose to the medium stimulated growth. Growth in the medium containing the heated TMAO-glutamate solution was inhibited for the first 12 hr of incubation; however, at 24 and 48 hr the absorbance was comparable to the control.

The TMAO-glucose solution and media heated with TMAO developed a yellow color and had a UV spectrum with an absorption peak at 270 nm. Song and Chichester (13) observed that both characteristics are indicative of a Maillard-Amadori reaction.

Gas chromatographic analysis of heated defined medium containing 1% TMAO showed peaks which corresponded to known trimethylamine (TMA) and DMA samples. The presence of DMA in heated defined medium plus 1% TMAO was indicated further by the melting point of the benzyl derivative crystals. The crystals had an uncorrected melting point of 40°C, compared to the published value of 41°C for the benzyl derivative of DMA (12).
Preliminary trials showed that autoclaved TYG broth containing 1% TMA did not inhibit growth of *B. megaterium*. However, growth of *B. megaterium* cells (100/ml) was completely inhibited in TYG broth heated with 1% DMA (Fig. 2). Subcultures of 10 ml of TYG prepared with 1 ml of the 1% DMA broth after 96 hr of incubation showed that viable organisms remained. At a concentration of 0.5% DMA heated in the medium, the lag phase was extended by approximately 16 hr, compared to that in normal broth, and by about 14 hr, compared to broth heated with 1% TMAO. The logarithmic growth rates were similar to these three cultures. A medium prepared by the addition of 1 ml of an autoclaved solution of 0.05 m DMA and 0.05 m glucose to 9 ml of autoclaved TYG was slightly more inhibitory than the TMAO containing broth. No inhibition was noted in a culture containing 1% DMA which had been autoclaved in 0.01 m sodium phosphate buffer before its addition to the medium.

A yellow to brown color was observed in media heated with DMA and in the heated DMA-glucose solution, indicating a Maillard-Amadori reaction.

**DISCUSSION**

With the autoclaving treatments (121 C) used in these experiments, TMAO could be reduced to TMA (11) or decomposed to DMA and formaldehyde (14). The presence of TMA and DMA in defined medium heated with TMAO shows that both reactions occurred. The data indicate that part of the DMA further reacted with glucose by a Maillard-Amadori or browning reaction to produce the inhibitor. Heating TMA in broth did not result in inhibition. It should be noted that TMA, a tertiary amine, would not participate in a browning reaction, a reaction between a primary or secondary amine and a carbohydrate. Similarly, TMAO would not be expected to form a Maillard-Amadori product.

As noted above, formaldehyde and DMA are produced in the decomposition of TMAO. It is doubtful that the formaldehyde contributed significantly to inhibition in media heated with TMAO. Formaldehyde is a low boiling compound, and it seems logical that it would have been volatilized during the 15 min of heating at 121 C. In addition, reacting DMA and glucose produced an inhibitor, and no formaldehyde would have been produced from these reactants.

The inhibitor formed by the reaction of DMA and glucose did not appear to have a lethal effect on cells of *B. megaterium*. Viable cells were recovered by subculturing 1 ml of TYG heated with 1% DMA after 96 hr of incubation in which no growth occurred, indicating that fewer than 100 cells/ml (the initial inoculum) were killed after 4 days of exposure to the inhibitor.

In most instances, the DMA-glucose reaction product extended the lag phase of growth but, when compared to control cultures, had little effect on the logarithmic growth rate. This suggests that *B. megaterium* inactivated the inhibitor and thus reduced the inhibitor concentration to a level where growth could proceed at an uninhibited rate.

Maillard-Amadori products have been shown to be inhibitory because the amine reactant was essential for growth and when compounded with glucose it was less available to the microorganism (3). In the present study, only glucose would be considered essential. Ostensibly, the low concentration of glucose in the two media used (0.1% in TYG, 0.18% in defined medium) would suggest that all glucose may have been in the form of a browning reaction product when DMA or TMAO were heated in the media. However, the addition of heated solutions of TMAO and glucose or DMA and glucose to media resulted in inhibition although the media contained the normal glucose concentration.

Glycosylamine is an early intermediate of the Maillard-Amadori reaction (8, 9). A few such products formed from amino acids and glucose have been shown to inhibit bacterial growth (3, 4, 6, 10). Therefore, it seems possible that the glycosylamine of DMA and glucose inhibited *B. megaterium*. However, the heat process used would encourage the production of a variety of browning reaction products. Thus, the inhibitor may have been formed subsequent to the production of the glycosylamine.

The necessity of heating TMAO to obtain inhibition of *B. megaterium* indicates that this compound is not a selective agent in fresh sea-
foods as initially thought possible. However, the participation of DMA in a Maillard-Amadori reaction is of interest. Previous studies have shown that an amino acid and glucose may react to yield compounds which affect microbial growth. Inhibition by the DMA-glucose reaction products shows that Maillard-Amadori products from glucose and amines other than amino acids also may influence growth of microorganisms.

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