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Toxicity of Methanethiol Produced by *Brevibacterium linens* toward *Penicillium expansum*

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Methanethiol production was studied in cultures of *Brevibacterium linens* ATCC 8377 grown upon three different media. *B. linens* did not produce methanethiol when grown on a medium lacking methionine but did produce methanethiol and methyl disulfide when grown on a defined medium containing methionine and an undefined medium containing Trypticase. Methanethiol concentrations produced by *B. linens* were capable of inhibiting germination of spores of *Penicillium expansum* NRRL 877 (toxic concentrations being above 0.33 ppm methanethiol).

The characteristic aroma and flavor of surface-ripened cheeses result in part from the proteolytic activity of microorganisms found in the surface smear of these cheeses. *Brevibacterium linens*, the dominant bacterium of the smear, produces proteolytic enzymes and is responsible for the pungent aroma associated with these cheeses (Boyavel and Desmazeaud, 1983; Parliment et al., 1982; Langhus et al., 1945). Grill et al. (1967) found that the surface smear of Trappist cheese was necessary for production of methanethiol from methionine. Methanethiol, hydrogen sulfide, and methyl thioacetate are important sulfur-containing compounds in the aroma of surface-ripened cheeses (Dumont et al., 1974, 1976; Parliment et al., 1982).

A notable aspect of Limburger, Trappist, and other surface-ripened cheeses is their inability to support mold growth (Grecz et al., 1959; Shih and Marth, 1972). Grecz et al. (1961, 1962) investigated the possibility that an antibiotic produced by *B. linens* was responsible for the resistance to molding; however, the antibiotic was not identified. More recent work conducted with brevibacteria from human skin determined these bacteria produced an antibiotic active against gram-positive and -negative bacteria (Ryall et al., 1981; Al-Admany and Noble, 1981). Ryall et al. (1981) postulated that methanethiol produced by brevibacteria may inhibit the growth of dermatophytic fungi. Whether a constituent of surface-ripened cheese aroma is responsible for the resistance to molding exhibited by surface-ripened cheeses has not been examined.

Volatile compounds produced by *B. linens* have been demonstrated to inhibit spore germination and mycelial growth of several mold species including several commonly reported on spoiled cheese (Beattie and Torrey, 1985), and inhibition of mold growth depended upon the presence of methionine in bacterial growth media. *Penicillium expansum*, the most resistant of molds tested, was selected as the test organism for this study. We report herein on the toxicity of methanethiol on spores of *P. expansum*, the rate of production of methanethiol by *B. linens*, and use of polyester/polyethylene laminate pouches as controlled atmosphere growth chambers.

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MATERIALS AND METHODS

Organisms and Culture Conditions. *B. linens* ATCC 8377 was obtained from the American Type Culture Collection, Rockville, MD, and *P. expansum* NRRL 973 was obtained from the Northern Regional Research Center, Peoria, IL. Malt agar (Difco Laboratories, Detroit, MI) supplemented with 3 g/L of yeast extract (MYE) was used for growth and maintenance of *P. expansum*. A medium (TYE) containing Trypticase (BBL Microbiology Systems, Cockeysville, MD; 10 g/L), yeast extract (Difco; 5 g/L), and agar (Difco; 5 g/L) was used for growth and maintenance of *B. linens*.

A defined medium (DMM), which was also used for bacterial growth, contained buffered mineral salts solution (Meynell and Meynell, 1970), amino acids (0.125 μg/mL each) alanine, aspartic acid, glutamic acid, lysine, methionine, and tryptophan (all Sigma Chemical Co., St. Louis, MO), and vitamins (per L) biotin (5 μg), folic acid (50 μg), and thiamine (1.0 mg) (all Sigma). Methionine could be excluded from the medium (DMA) without affecting bacterial growth. When required, media were solidified with 15 g of Noble agar (Difco) per liter. The pH of each medium was 7.0.

Bacterial inocula were prepared in DMA broth; these cultures were incubated aerobically at 30 °C for 12-18 h. Petri plates containing TYE, DMM, or DMA agar were inoculated by spreading 0.5 mL of the broth culture of *B. linens* on the agar surface. Inoculated plates were placed in flexible pouches (16.2 cm × 24.4 cm) composed of polyester (0.5 mil)/polyethylene (1.5 mil) laminate (Dazey Corp., Industrial Airport, KS). Oxygen-transmission rates for this laminate were in the range 11.9-27.1 cm^3/m^2 for 24 h as determined by the manufacturer using American Society for Testing and Materials Method D-3985-81

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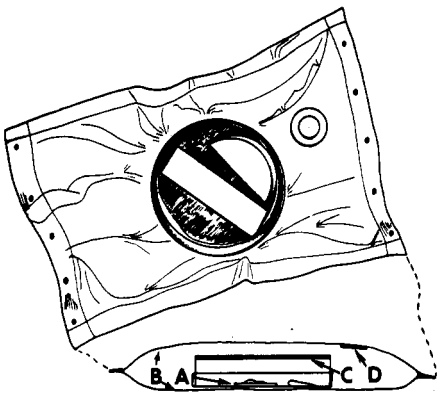

Figure 1. Diagram of a flexible laminate growth chamber: A, slide culture; B, growth chamber; C, bacterial culture; D, septum sealed into growth chamber wall to allow sampling of growth chamber atmosphere.

(ASTM, 1984). A Teflon/rubber septum coated with silicon high-vacuum grease was sealed into a pocket on the interior wall of each pouch. Pouches were closed by means of a heat sealer (Dazey Corp.) to form growth chambers. The positions of mold and bacterial cultures within growth chambers are illustrated in Figure 1. Samples of the atmospheres from growth chambers were taken through the septum by means of a gas-tight syringe. Growth chambers were incubated at 25 °C.

Analyses of Growth Chamber Atmospheres. Samples of atmospheres were injected directly into a Varian 3700 gas–liquid chromatograph (Varian Instruments, Palo Alto, CA) equipped with a flame ionization detector, on-column injection, and a glass column (1.83 m × 0.63 cm o.d.) packed with 10% DC-200 on Chromosorb W(AW) as described by Leppanen et al. (1980). The detector and injection temperatures were, respectively, 200 and 180 °C, and the oven was programmed (without delay) from 40 to 110 °C at 8 °C/min and equipped with flame ionization detector. Nitrogen was used as carrier gas at a flow rate of 30 mL/min. To determine the tentative identity of compounds occurring in atmospheres of growth chambers, retention times for peaks occurring in sample chromatograms were compared with retention times of peaks in chromatograms of standards. Methanethiol (Eastman Kodak, Rochester, NY), methyl disulfide (Aldrich Chemical Co., Milwaukee, WI), and methyl thioacetate (Alfa Products, Danvers, MA) were used as standards. Retention times of standards were 0.71, 3.36, and 2.27 min, respectively.

Stock mixtures of methanethiol in nitrogen gas were made in empty sealed pouches. Evacuated pouches were reinflated with nitrogen gas and then injected, via a gas-tight syringe, with a known weight of liquid methanethiol. Dilutions were made at room temperature from this stock mixture into pouches containing nitrogen. Concentrations of methanethiol in growth chambers were determined from a standard curve correlating peak height from chromatograms with concentration.

Spore Germination Assay. An adaptation of the slide-germination method of the American Phytopathological Society (1943) was used to evaluate the influence of methanethiol on germination of spores of *P. expansum*. A drop of MYE on a sterile microscope slide was inoculated with 50–100 mold spores; this slide culture was placed into an inverted Petri plate. Moistened filter papers were added to the Petri plate to provide adequate humidity. The slide culture and Petri plate were then sealed into a growth chamber. Atmospheres in sealed growth chambers were removed by means of a gas-tight syringe. A known volume of air was injected into the collapsed growth chambers using a gas-tight syringe. Methanethiol from stock mixtures was then injected into growth chambers to yield concentrations of 0.16, 0.33, 0.82, 1.6, and 3.3 ppm. Growth chambers with no methanethiol were used as controls.

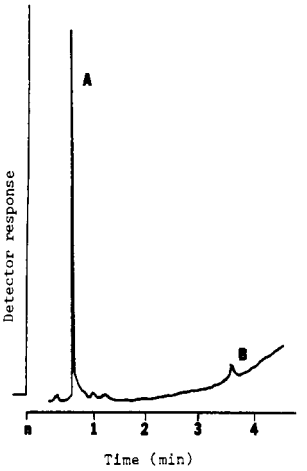

Figure 2. Chromatogram of atmosphere from growth chamber containing *Brevibacterium linens* ATCC 8377 grown upon DMM medium for 84 h. Attenuation 32× at 10^{-12} sensitivity. Key: A, methanethiol; B, methyl disulfide.

Growth chambers were incubated at 25 °C. Chambers containing 0, 0.16, and 0.33 ppm methanethiol were opened after 24-h incubation because outgrowth of spores was observed. Germinated spores were counted by means of a microscope. Remaining chambers were opened and examined after 48-h incubation.

RESULTS AND DISCUSSION

We have observed previously (Beattie and Torrey, 1985) that mold spores representing four species did not germinate when incubated in growth chambers containing strains of *B. linens* grown upon TYE or a defined medium that contained methionine (DMM). Disposable anaerobic indicators (BBL Microbiology Systems) placed in growth chambers demonstrated that sufficient oxygen was present to prevent change of the methylene blue indicator to colorless. Spores did germinate in chambers containing *B. linens* grown on medium from which methionine was absent (DMA). These observations indicate that the mold is inhibited by a metabolite of methionine. Comparison of odors from growth chambers in which *B. linens* was incubated on DMM or DMA media indicated presence of a volatile sulfur compound in atmospheres over DMM but not over DMA.

Periodic gas chromatographic analyses were done on samples from atmtospheres in growth chambers containing *B. linens* growing upon TYE, DMM, and DMA media. Methanethiol was not detected in atmospheres when *B. linens* was grown on DMA, a medium that lacked methionine. However, when samples from chambers containing *B. linens* grown on media including protein (TYE) or methionine (DMM) were chromatographed (Figure 2), methanethiol and methyl disulfide were detected but not methyl thioacetate. Methyl disulfide, probably from oxidation of methanethiol (Parliment et al., 1982), was detected only in trace amounts at incubation times of 84 h and longer. Since these incubations were longer than the 48 h necessary to develop fungal toxicity in growth chambers (Table I), and since methyl disulfide is less toxic to fungi than methanethiol (Owens, 1969; Lewis and Papavizas, 1971), these traces of methyl disulfide were considered unlikely to influence spore germination.

Table I. Effect of Methanethiol Concentration on Germination of Spores of *Penicillium expansum* NRRL 973

methanethiol concn, ppm	no. of spores germin[b]	methanethiol concn, ppm	no. of spores germin[b]
0.0[a]	107 ± 5	0.82	0
0.16	107 ± 6	1.6	0
0.33	86 ± 46	3.3	0

[a] Control, no methanethiol added. [b] Mean of three replicates ± standard deviation.

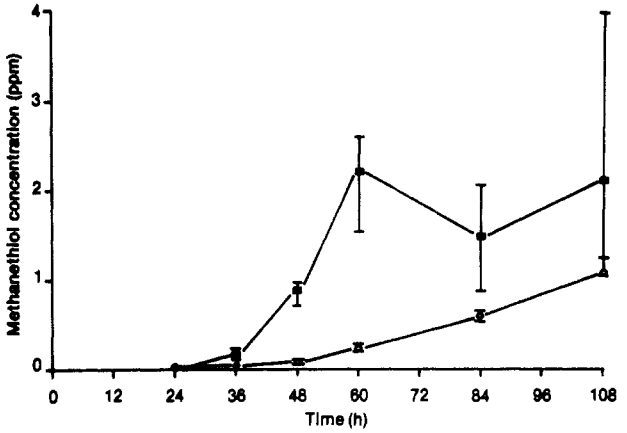

Figure 3. Methanethiol production by *Brevibacterium linens* ATCC 8377 grown upon DMM (■) and TYE (●) media, average of three replications.

Concentrations of methanethiol in growth chambers were determined at intervals during incubation (Figure 3). Data from growth studies in this laboratory demonstrated that toxic concentrations of methanethiol occurred in growth chambers after 24–36-h incubation of *B. linens* on DMM and TYE media. Examination of Figure 3 reveals that methanethiol concentrations on both media during this period ranged from about 0.2 to 1.2 ppm.

Spores of *P. expansum* were used to correlate concentration of methanethiol with toxicity. A series of growth chambers containing slide cultures of spores was injected with various concentrations of methanethiol, and the chambers were then incubated. Spores were killed by the presence of 0.82 but not 0.33 or 0.16 ppm methanethiol (Table I).

Cuer et al. (1979) have reported methanethiol, hydrogen sulfide, methyl disulfide, and methyl thioacetate in volatiles from strains of *B. linens* cultured on a medium containing protein. Methanethiol produced during decay of cruciferous plants inhibited growth and zoospore germination (Lewis and Papavizas, 1970, 1971). Owens (1969) has suggested that reaction of methanethiol with sulfhydryl groups may cause inactivation of enzymes necessary for germination.

Results from our laboratory indicate that a volatile metabolite produced from methionine by *B. linens* is responsible for inhibition of mold growth and spore germination. We report herein the identification of methanethiol and dimethyl sulfide as the major sulfur-containing compounds detected in atmospheres of growth chambers in which *B. linens* was cultured on media containing methionine (DMM, TYE). Concentrations of methanethiol increased during incubation to maxima for TYE (4.0 ppm) and DMM (1.13 ppm) at 108 h. Concentrations of methanethiol less than 1 ppm were capable of inhibiting germination of spores of *P. expansum*. Two topics we are pursuing based on results presented here are (a) evaluation of the antimycotic activity of methanethiol produced by *B. linens* on surfaces of semisoft cheese and (b) the potential for methanethiol to prevent mold growth on other cheeses.

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