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Identification of Volatile Compounds Produced by Brevibacterium Linens that Inhibit Molds

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IDENTIFICATION OF VOLATILE COMPOUNDS PRODUCED BY

Brevibacterium linens THAT INHIBIT MOLDS

BY

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IDENTIFICATION OF VOLATILE COMPOUNDS PRODUCED BY

Brevibacterium linens THAT INHIBIT MOLDS

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

George S. Torrey   Date
Thesis Advisor

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Knowing others is wisdom;

Knowing the self is enlightenment.

Mastering others requires force;

Mastering the self needs strength.

He who knowns he has enough is rich.

Perseverance is a sign of will power.

He who stays where he is endures.

To die but not to perish is to be eternally present.

Lao Tsu, 600 B. C.

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TABLE OF CONTENTS

TABLE OF CONTENTS
(continued)

LIST OF TABLES

List of Tables
(continued)

LIST OF FIGURES

Figure   Page

Article I

Article II

Appendices

LIST OF FIGURES
(continued)

INTRODUCTION

Mold contamination of processed food and its raw ingredients is a major concern in agriculture. Although at one time mold contamination of cheese and other foods was considered to be of little consequence, discovery of carcinogenic and/or teratogenic toxins (73) produced by molds commonly associated with food has changed this innocuous and frequent inconvenience into a serious health consideration.

The presence of mold on or in cheese is a common occurence, indeed, many cheeses depend upon molds to provide characteristic cheese flavor and texture. Cheeses such as blue, Roquefort, Camembert, and Brie are ripened under controlled conditions by species of Penicillium.

While certain molds are desirable, adventitious molds are undesirable contaminants in manufacture of most cheeses. Long ripening periods at controlled temperatures and humidity favor the growth of adventitious molds on Cheddar and other hard cheeses. Control of molds in ripening rooms and throughout cheese manufacture is important. One mold colony can give rise to thousands of airborne spores which can contaminate many kilograms of cheese. Some of the molds isolated from cheese produce mycotoxins in the cheese (10, 11, 48); therefore, control of mold growth is of paramount importance for the cheese industry.

Surface-ripened cheeses are ripened by a smear of

bacteria and yeasts growing on the surface of the cheese. Brevibacterium linens, the predominant microorganism in the smear of surface-ripened cheeses, is responsible for the typical aroma and flavor of Limburger, Trappist, Brick, and similar cheeses (1, 5, 8, 41, 42, 43, 60, 84). The aroma of these cheeses is a well-known characteristic and is due to the proteolytic metabolism of microorganisms in the surface smear (1, 2, 13, 21, 22, 23, 24, 26, 81, 84, 85).

An interesting aspect of surface-ripened cheeses noted by Grecz et al. (29) is resistance to spoilage by bacteria or molds. These workers found that extracts of surface-ripened cheeses and of B. linens produced similar antimicrobial activity (30, 31). Grecz et al. did not examine the aroma of surface-ripened cheeses for antimicrobial activity. The aromas of surface-ripened cheeses contain a variety of compounds, several of these compounds are inhibitory to mold growth in environments other than cheese.

The purpose of research presented in this thesis was to evaluate volatile compounds produced by B. linens for antimycotic activity. A method was developed to quantify inhibition of mold growth and spore germination in the presence of B. linens. The identity of the inhibitory compound was determined. Furthermore, the concentration of the inhibitory compound necessary to inhibit spore germination was determined.

This thesis is arranged in an alternative style. The comprehensive literature review is followed by two journal articles which describe the majority of materials and methods. Results and discussion are also included in the journal articles. Materials and methods or results not presented in an article are placed in appendices as are statistical information and raw data. The conclusions derived from the project are included as a separate section.

LITERATURE REVIEW

The microorganism most responsible for ripening of surface-ripened cheeses is Brevibacterium linens; this microorganism has a significant role in development of flavor, aroma, and texture in Limburger, Trappist, and Leiderkranz cheeses. Knowledge of the microbial succession occurring on the surface of this type of cheese and how the ecology of the cheese relates to organoleptic properties of the cheese is necessary to understand the antimycotic phenomena exhibited by these cheeses. The presence of sulfur-containing compounds in the aroma of cheese and production of these compounds by the surface microflora provide insight to the antimycotic activity. Furthermore, examination of cheese spoilage and methods to prevent mold spoilage of cheese is desirable to understand the need for new antimycotic agents. Finally, this literature review would not be complete without an overview of surface-ripened cheese manufacture.

Brevibacterium linens

Bacterium linens was first described by Wolff (as cited in 9, 21) as a short gram-positive rod that produced a red-brown pigment and was responsible in part for the slime on surface-ripened cheeses. Steinfatt (as cited in 21) provided a more detailed description of the bacterium as a gram-positive, aerobic, nonsporeforming, nonmotile rod.

The bacterium liquefied gelatin and produced an alkaline reaction in milk after 10 days of incubation.

Breed (as cited in 70) in 1963 proposed changing the name of Bacterium linens to Brevibacterium linens to define more clearly the genus Bacterium. The seventh edition of Bergey's Manual of Determinative Bacteriology placed the genus Brevibacterium in the family Brevibacteriaceae and classified B. linens as the type species for the genus (9). B. linens was described as a gram-positive, short, unbranched, gram-positive rod that gave a positive catalase test, liquefied gelatin, and was salt tolerant.

The coryneforme morphology exhibited by B. linens was not described in the seventh edition of Bergey's Manual of Determinative Bacteriology; however, Schefferle (as cited in 21) had observed snapping cell division and palisade cell arrangements, typical of corynebacteria, in cultures of B. linens. Mulder and Antheunisse (58) examined 150 strains of Arthrobacter, a genus of bacteria with coryneforme morphology, isolated from soil, activated sludge and dairy products and compared these isolates with B. linens and bacteria of other genera. These workers found Arthrobacter strains isolated from soil and activated sludge quite similar to each other yet different from strains isolated from dairy products. Furthermore, some of the strains isolated from dairy products were probably identical to B. linens. B. linens and these dairy strains were similar in nutritional,

morphological, and gram-staining characteristics. The pleo-
morphic tendencies of isolates of Arthrobacter and B. linens
were also noted. Pleomorphism was most pronounced as the
cultures aged or when incubation was in minimal medium.
Arthrobacter globiformis was found to be very similar to B.
linens.

Mulder et al. (59) further compared B. linens and
Arthrobacter strains isolated from cheese. Two types of
coryneforme bacteria were isolated from cheese; one forming
grey-white colonies, the other forming orange colonies. B.
linens and the orange colony-forming bacteria were similar
morphologically and nutritionally. Strains of both types
were able to grow within 31 days at 30 ^{o}C in media containing
up to 15% sodium chloride. Both types, in agreement with
studies on Limburger and similar cheeses (1, 2, 13, 21, 22,
23, 24, 26, 81, 84, 85), were proteolytic. Because of the
similarities between the orange-colony forming bacteria and
B. linens, Mulder et al. (59) concluded that the pigmented
bacteria should not be placed in the genus Arthrobacter.

The similarities among strains of Arthrobacter, B.
linens, and other coryneforme bacteria have been examined on
the basis of 57 characteristics (16). A. globiformis and B.
linens were found to be very similar; 84% of the character-
istics were properties of both microorganisms. This
prompted daSilva and Holt (16) to propose that the name of
B. linens be changed to "A. linens (Wolff) daSilva and Holt"

and that other species in the genus <u>Brevibacterium</u> be
examined for similarities to other genera. The eighth edi-
tion of <u>Bergey's</u> <u>Manual</u> <u>of</u> <u>Determinative</u> <u>Bacteriology</u> lists
all species of brevibacteria as <u>species</u> <u>incertae</u> <u>sedis</u> (70),
indicating <u>Brevibacterium</u> may not be a discrete genus and
individual species may belong in other genera.

<u>Microbial</u> <u>ecology</u> <u>of</u> <u>surface-ripened</u> <u>cheese</u>

The observed softening of surface-ripened cheese
during ripening because of growth of <u>B</u>. <u>linens</u>, indicates
that the organism produces proteolytic enzymes. Albert et
al. (5) found that pure cultures of <u>B</u>. <u>linens</u> were proteo-
lytic in skim milk. An increase in soluble nitrogen in
cultures was noted for a period of up to 81 days after
inoculation. These workers also found an alkaline reaction
in litmus milk with slight digestion of the medium observed
10 days post-inoculation and complete digestion of the
medium in several weeks. The cultures were not ferment-
ative.

The presence of yeasts on the surface of young
surface-ripened cheeses was found to be important in the
normal development of the cheese (36, 39, 40, 42, 67, 81,
84). Kelly (39), using microscope slide pressings from the
surface of Limburger cheese, followed the succession of
microorganisms over time. Initially, the dominant group of
microorganisms occurring on the surface of the cheese was
yeasts. Coincident with lysis and subsequent disappearance

of yeasts was appearance of short gram-positive rods. Kelly and Marquardt (40) found that the surface pH of the cheese increased from 4.9 to 5.8 in the eight days that yeasts were present. B. linens was found to be incapable of growth at pH lower than 5.8, and was not able to grow on fresh cheese curd having a low pH. These authors concluded that yeasts were able to metabolize lactic acid and proteins, raising the pH of the cheese to 5.8 and above, thereby providing conditions suitable for growth of B. linens.

While increasing the pH of the cheese surface aided growth of B. linens, yeasts also provided necessary growth cofactors (21, 36, 67, 84). Purko et al. (67) found that pure cultures of yeasts isolated from Limburger cheese were able to produce pantothenic acid, riboflavin, biotin and niacin. These yeasts were able to increase the growth of B. linens in a vitamin-free medium. Yeasts isolated from sur-face-ripened cheeses were identified as species of Mycoderma and Torula (21, 81, 84). Later work by Cone and coworkers (2, 81, 85) identified the major proteolytic yeasts occurring on surface-ripened cheese as species of Trichosporon.

Proteolytic activity of yeasts isolated from Limbur-ger and Brick cheese appeared to be of minor nature with the major portion of proteolysis having occurred in the cheese after the yeasts had been replaced by bacteria or in 8-10 days (21, 39, 42). Amounts of free amino acids increased as the proteolytic bacteria digested the cheese. Methionine,

lysine, and glutamic acid were liberated in the greatest quantities from the proteins. The largest increase in free amino acids was found between 9 and 14 days post salting of the cheese (21).

Yeasts associated with Trappist cheese produced endocellular proteinases, polypeptidases, and peptidases and were important in increasing free amino acids in the cheese (2, 81, 85). Cheese ripened with yeast and B. linens had a higher free amino acid content through 35 days of ripening than cheese ripened with B. linens alone. An extracellular aminopeptidase from B. linens was isolated and characterized by Foissy (22, 23, 24). The pH optimum for the enzyme was 9.6, somewhat higher than the pH at the surface of the cheese during ripening (23). Torgeson and Sorghaug (82) found five enzymes that were able to hydrolyze a variety of dipeptides. An additional enzyme was found that was very active in peptide hydrolysis. The authors conclude that this enzyme was possibly a proteinase rather than a peptidase. Lysis of cells during starvation or for other reasons would release these enzymes into the cheese furthering proteolysis. An extracellular proteinase identified by Freidman et al. (26) was specific for casein, especially β-casein, and had a pH optimum between 7.2 and 7.3. Release of proteolytic enzymes by B. linens progressively degrades cheese proteins, increasing soluble nitrogen with age as peptides are formed (21). Textural and flavor changes occur with

enzymatic degradation of casein.

Importance of sulfur-containing compounds in aroma of cheeses

The aroma and flavor of a cheese is dependent upon many factors, most important being the components of the cheese and the microbial growth on or in the cheese (1, 60, 69, 88). Compounds important in cheese aroma are produced as by-products of microbial growth or are produced by chemical reactions facilitated by conditions created by bacterial growth. The protein and fat portions of cheese are substrates which microorganisms use readily to produce important and characteristic aroma compounds. For example, the aroma of blue cheese varieties is derived from the presence of methylketones (1, 62). Methylketones are catabolic end-products of lipolytic molds associated with blue cheeses (1, 62).

Enzymatic degradation of cheese proteins by microbial enzymes yield compounds which have been found to be important in the aroma of surface-ripened and Cheddar cheeses (32, 33, 44, 49). Early work on the aroma of Cheddar cheese detected sulfur-containing compounds. Removal of these sulfur compounds by chemical traps altered the aroma of the cheese (47, 50, 52, 53, 86). Dateo et al. (17) devised a gas entrainment system for volatile sulfur compounds produced by cooking cabbage. This entrainment system found use in studying cheese aromas. The entrainment system

was composed aqueous solutions of lead acetate, mercuric cyanide, and mercuric chloride (Table 1). Walker (86) used cadmium sulfate in place of lead acetate to remove hydrogen sulfide and thiols in the presence of moisture. These trapping compounds coupled with aroma or taste panels were useful in determining which sulfur compounds were the most important constituents of Cheddar cheese aroma. The aroma was most altered after passing through trapping compounds that removed thiols and hydrogen sulfide (47, 50, 52, 53, 86).

Methanethiol, the most important constituent in the aroma of Cheddar cheese (52, 53), and other volatile sulfur compounds are derived from sulfur-containing amino acids present in cheese proteins (38, 43, 52, 53). Formation of methanethiol from methionine by adventitious and starter bacteria is not a major source of methanethiol in Cheddar cheese. The starter cultures employed in Cheddar cheese manufacture do not have this capablity and conditions in the cheese during aging do not favor growth of adventitious microorganisms (44, 74, 75). Law and Sharpe (44) hypothesized that the reducing environment produced by the starter culture favors nonenzymatic production of methanethiol from low molecular weight precursors produced by the starter culture. Furthermore, cheese made with gluconic acid lactone did not contain methanethiol and had a more positive redox potential (44, 50, 51).

Table 1. Chemical traps employed to remove sulfur-containing
compounds form atmospheres in growth chambers.

Trap	Concentration of trapping agent	Compounds trapped
Mercuric chloride	3% (W/V)	Thiols, sulfides, disulfides
Mercuric cyanide	4% (W/V)	Thiols, hydrogen sulfide
Cadmium sulfide	5% (W/V)	Hydrogen sulfide

Methionine is necessary for production of methanethiol by pseudomonads and corynebacteria associated with cheeses (1, 33, 38, 44, 72, 74). Segal and Starkey (72) proposed that methionine is degraded to methanethiol by the following method:

oxidative deamination · · · · · · · demethiolation

methionine → α-ketomethionine + ammonia (NH$_3$) → α-ketobutryic acid + methanethiol (CH$_3$SH)
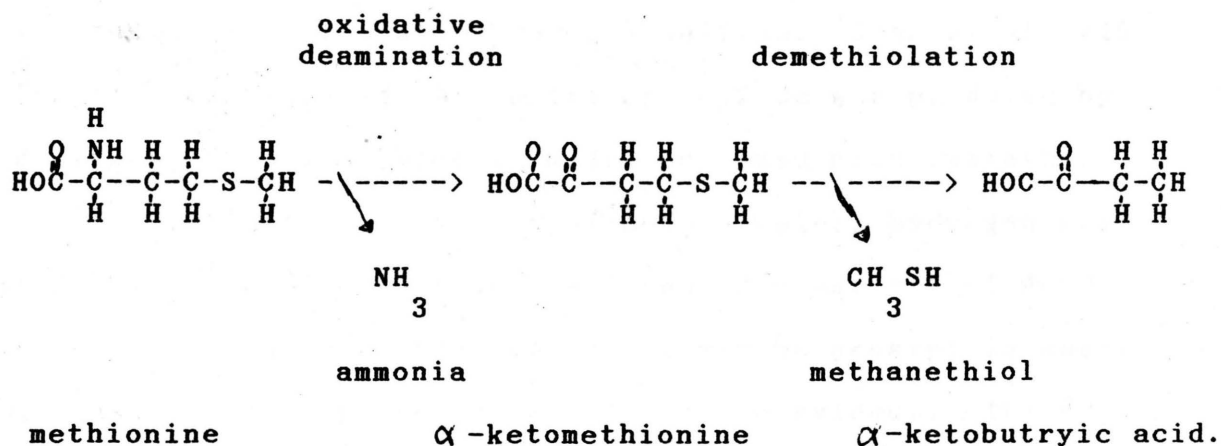
Deamination and demethiolation are enzyme-mediated reactions. Some bacteria oxidize methanethiol to produce methyl disulfide and methyl sulfide which are also important in cheese aroma (38, 72). Methanethiol production from methionine labelled with ^{35}S demonstrated that the amino acid was demethiolated by the bacterium associated with Trappist cheese, i. e. B. linens (38). Grill et al. (33) used an entrainment similar to that of Dateo et al. (17) and trapped sulfur-containing compounds derived from the degradation of isotopically marked methionine. In traps specific for hydrogen sulfide little radioactive material was found when compared to traps specific for methanethiol. Grill et

al. (33) hypothesized that sulfur-containing compounds other than methionine were precursors to hydrogen sulfide. Kadota and Ishida (38) reviewed several precursors to hydrogen sulfide production by bacteria. Cysteine and cystine, both present in casein and other proteins of milk and cheeses, are common precursors of hydrogen sulfide. Cuer et al. (15) further demonstrated that hydrogen sulfide was produced by B. linens when incubated in media enriched with cysteine.

Small concentrations of methanethiol, hydrogen sulfide, and methyl disulfide are found in a variety of food products. Although these compounds may be present in small quantities their presence is often quite evident. The odor thresholds of these sulfur compounds are low: methanethiol, 0.02 ppb; hydrogen sulfide, 0.18 ppb; dimethyl sulfide, 0.33 ppb (6, 61). Jansen found that the taste threshold for these compounds in beer ranged from 1.0 ppm for methanethiol to 10.0 ppm for methyl disulfide (37). Taste and odor thresholds are different; odor thresholds are approximately 10,000 times lower than taste thresholds (6).

More recent studies on the aroma of surface-ripened cheeses have relied upon more sensitive and accurate separation techniques using mass spectrometry to identify compounds (19, 20, 64). Vacuum distillation of extracts from several surface-ripened cheeses yielded over sixty neutral compounds (19) and in further work on Pont l'Eveque, a surface-ripened cheese, 123 compounds were identified (20).

Methanethiol, methyl disulfide, and thioesters were identi-
fied (20). This was the first evidence of the production of
thioesters by bacteria associated with cheeses. S-methyl-
thioacetate, thought to be formed by enzymatic acylation of
methanethiol, has a low odor threshold, 5 ppb, and an odor
similar to that of cooking cauliflower (1, 64). Thioesters
are important in off flavor development in beers and other
fermented beverages (37, 54, 55), and may be significant
constituents in the aroma of surface-ripened cheese (19, 20,
64).

Spoilage and preservation of cheese

Manufacturing milk into cheese is an efficient
method of concentrating milk constituents and preserving
them from microbial spoilage. Many microorganisms, patho-
genic and nonpathogenic, find milk a source of readily
available nutrients, thus preventing long term storage of
milk. Cheese has several characteristics which make it less
susceptible to spoilage by microorganisms; (a) low pH
because of acid production by starter cultures (41, 60);
(b) salt concentration (1 to 2%) inhibitory to growth of
spoilage organisms; (c) reduced oxidation/reduction
potential making growth difficult for aerobic microor-
ganisms; (d) inhibitory compounds produced by starter cul-
tures (10, 35).

Cheese will, however, spoil if proper precautions

are not taken during manufacture and storage. Spoilage of cheese may vary from a slight flavor defect that is detectable only by experienced cheese graders to gross defects such as holes in the cheese or bloated packages caused by gas-producing microorganisms (13, 41, 60, 69, 88). Preventing spoilage of cheese by adventitious microorganisms is best accomplished through maintaining sanitary conditions during cheese manufacturing and using an active starter culture.

Perhaps the major type of spoilage found with cheese is surface mold growth. Species of Penicillium and Aspergillus are the most common molds found on Cheddar and Swiss cheese (10, 11). Unlike many bacteria, molds can grow at low pH and temperature causing serious problems during aging and storage of cheese. The amount of cheese discarded or downgraded because of mold growth has not been estimated.

Mold contamination of foods is aesthetically unpleasant, and, with the discovery of mycotoxin production by molds commonly associated with foods, mold contamination has been recognized as a serious health hazard. Mycotoxins are produced by a variety of molds, including species of Aspergillus, Penicillium, Alternaria, and Fusarium (34, 79); all of these molds have been isolated from cheeses (10, 11). Perhaps the most economically important mycotoxin-producing molds are species of Aspergillus especially A. parasiticus and A. flavus which produce aflatoxins (10, 11, 34, 73).

Aflatoxins are among the most carcinogenic and toxic compounds yet identified (73). Somewhat less toxic are toxins produced by species of Penicillium; these toxins include penicillic acid, ochratoxin A, and patulin (66, 79).

The production of mycotoxins by molds depends upon the substrate on which the organism is growing (34, 66). Molds isolated from cheeses are able to produce a host of toxins including aflatoxins, patulin, and penicillic acid (10, 11). Aflatoxins can be produced by A. flavus and A. parasiticus growing on cheese, but more importantly, afla-toxins can diffuse into the cheese to a depth where simply removing the visible mold may not remove all of the myco-toxin (25, 48, 76). This feature of mold contamination has serious health considerations.

Cheese is a unique food because of the long aging time many cheeses require before use. Cheeses made from unpasteurized milk are required by law to have at least 60 days aging to reduce health risks inherent in consuming raw milk. The long aging period necessitates use of antimycotic agents to reduce surface mold growth.

The most commonly used antimycotic agents for cheese preservation have been salts of sorbic acid (2, 4-hexadi-enoic acid) (68, 77). Potassium sorbate is used because sorbic acid, a polyunsaturated organic acid, is sparingly soluble in water. At 20°C, only 0.16 g sorbic acid will dissolve in 100 ml water, whereas 139 g potassium sorbate

will dissolve in 100 ml water (12). Organic acids, e. g.
sorbic, propionic, and lactic acids, are most effective as
antimicrobial agents in the undissociated form. Sorbic acid
is most effective in low acid foods (12, 77). Sofos and
Busta (77) state that sorbates are effective against molds
at levels of 0.05-0.3%. Both potassium sorbate and sorbic
acid are generally recognized as safe (GRAS) by the Food and
Drug Administration (FDA) (68).

Application of sorbic acid to cheese may be by sev-
eral methods including spraying or dipping, dusting the
cheese surface with sorbic acid powder, or impregnating the
compound into the packaging material (77). Sorbic acid has
a strong odor and flavor that may impart an off-flavor to
cheese. Moreover, sorbic acid can be metabolized by species
of Penicillium to yield a compound that will impart a hydro-
carbon off-flavor to the cheese.

Another organic acid which has been used as a cheese
preservative is propionic acid (12, 18). As with other
organic acids, propionic acid and salts of propionic acid
are most effective against molds in the undissociated
form, therefore, propionic acid is most effective in foods of
low pH (18). By law, the upper limit for direct addition of
propionic acid to cheese and cheese products is 0.3% (18);
propionate may also be added to the packaging material.
Propionic acid and its salts are GRAS. The compound has a
strong odor and flavor and is an important component in the

aroma of Swiss cheese (12, 18).

In 1982 the FDA approved limited use of natamycin (previously known as pimaricin), an antibiotic produced by Streptomyces natalensis, as an antimycotic on cheese cuts and slices (28, 68). The use of natamycin on Italian and natural cheeses, including Swiss, Cheddar, and blue was approved in 1984 (28). The antibiotic was proven to be effective against mold growth at concentrations as low as 50 ppm, however, natamycin does not affect bacteria (28, 68). FDA regulations allow spraying or dipping cheese in aqueous solutions containing 200-300 ppm natamycin (14). Natamycin may be incorporated into the packaging material either as a constituent of packaging film (0.1% natamycin) or as an edible coating material containing 0.2% natamycin.

Antimycotic agents produced by bacteria

Certain volatile sulfur compounds are known anti-mycotic agents (3, 27, 45, 46, 56, 57, 63, 65, 80). The ecology of various environments, including cheeses, is influenced by the presence of bacteria producing antimycotic agents during the decomposition of a substrate. Microorganisms may also produce nonvolatile antimicrobial substances such as antibiotics and toxins which aid the microorganism in competing for available nutrients.

The antimicrobial activity of surface-ripened cheese was first noted by Grecz et al. (29). In a series of experiments these workers demonstrated that growth of Clostridium

<u>botulinum</u> and species of <u>Penicillium</u> was inhibited when these organisms were inoculated onto sections of surface-ripened cheese. Inhibition of molds occurred on cheese at least eight weeks old that had been heated at 121° C for 15 min. Cheeses aged less than eight weeks were less inhibitory to growth of molds and <u>C</u>. <u>botulinum</u> (29). Extracts of the cheese were further examined for antimicrobial activity. Growth of <u>C</u>. <u>botulinum</u>, <u>Staphylococcus</u> <u>aureus</u>, and <u>Bacillus</u> <u>cereus</u> was inhibited by extracts of Leiderkranz cheese (30, 31). Finally, cultures of microorganisms isolated from surface-ripened cheese were examined for antimicrobial activity against <u>B</u>. <u>cereus</u> and <u>C</u>. <u>botulinum</u>. Antimicrobial activity was produced by mixed cultures only when cultures were aerated, and pure cultures of <u>B</u>. <u>linens</u> were found to exhibit antimicrobial activity (30). Because of the similarities in characteristics, the antimicrobial agent isolated from cheese and the antimicrobial agent isolated from <u>B</u>. <u>linens</u> were thought to be related antibiotics (31).

Weckbach and Marth demonstrated a reduction in toxin production by <u>A</u>. <u>parasiticus</u> when grown in a competetive environment with <u>B</u>. <u>linens</u> (87). Culturing <u>A</u>. <u>parasiticus</u> in the presence of <u>B</u>. <u>linens</u> reduced growth of mycelium, although the bacterium could no longer be isolated from the culture after three days of contact with the mold. The authors concluded that aflatoxins were toxic to <u>B</u>. <u>linens</u>.

Production of an antibiotic by brevibacteria asso-
ciated with environments other than cheese has been demon-
strated (4, 71). Brevibacteria isolated from human skin
were able to produce an antibiotic effective against S.
aureus (4). Ryall et al. (71), using synthetic skin medium,
showed that brevibacteria were able to inhibit growth of
fungi associated with tinea pedis (athlete's foot).
These workers hypothesized that methanethiol produced by the
bacteria was responsible for the observed inhibition. Pro-
teolysis of proteins in the synthetic skin by enzymes pro-
duced by the bacteria released methionine which was then
degraded to methanethiol.

Methanethiol and other sulfur compounds are produced
during ripening of Cheddar and surface-ripened cheeses.
These sulfur compounds may be found during degradation of
substances other than cheese. Cabbage, kale, and Brussels
sprouts (crucifers) release methanethiol, methyl disulfide,
and methyl sulfide during microbial degradation in soil
(45). The presence of crucifer stem and leaf tissue in
soils reduced the amount of pea root rot caused by Aphano-
myces euteiches. Further studies using pure sulfur-con-
taining compounds, including methanethiol, methyl disulfide,
and methyl sulfide, replicated inhibition of A. euteiches
zoospore germination, hyphal growth, and zoospore motility
(46, 63). Methanethiol when added to the headspace of
growth jars completely inhibited the growth of the fungi at

a level of 10 to 30 ppm methanethiol.

Sulfur is an important part of many fungicides and exhibits fungicidal activity in the elemental form (3, 95). The reactivity of the sulfhydryl group is important for the biological activity of thiols and some sulfhydryl-containing fungicides, although the exact mode or site of toxicity is unknown. Several possible reactions leading to thiol toxicity in biological systems were outlined by Torgeson (83):

1. Interactions with disulfide bonds in enzymes, thereby preventing the function of the enzyme. Thiols could also covalently bond with sulfhydryl groups in enzymes which rely upon a sulfhydryl group to function, e. g. acetyl Coenzyme A.

2. Chelating metal ions in cytochrome and other metal-lo-enzymes necessary for respiration. Hydrogen sulfide and short chain thiols are toxic to humans, part of this toxicity is due to the chelation of the metal ion in cytochrome oxidase (78).

3. Production of free radicals in the cell.

The presence of sulfur-containing compounds, such as methanethiol and hydrogen sulfide, in the aroma of surface-ripened cheese is well documented (1, 8, 13, 19, 20, 32, 33, 38, 44, 47, 49, 50, 51, 52, 60, 75, 86). While these compounds are toxic to humans in high concentrations, sur-face-ripened cheeses do not contain concentrations suffi-

cient to be harmful to humans. The resistance to molding exhibited by surface-ripened cheese may be in part because of toxic concentrations of sulfur-containing compounds produced by microorganisms, especially B. linens, responsible for ripening of this type of cheese.

Surface-ripened cheese production

Production of surface-ripened cheese is similar to that of other cheeses up to the point of whey draining and matting of the cheese curd (41, 60). Unlike Cheddar and other hard cheeses, most surface-ripened cheeses are salted after pressing by placing in a brine maintained at 22% sodium chloride. This method of salting also inhibits growth of undesirable microorganisms. Halophilic yeasts, present in the brine, are inadvertently inoculated onto blocks during handling and brining. These yeasts are important in providing conditions suitable for bacterial growth.

Typically, surface-ripened cheeses are produced in small-sized blocks, not larger than five pounds and as small as four ounces (41, 60). Limburger and other surface-ripened cheeses rely upon microorganisms growing on the cheese surface for flavor development. Small blocks with an actively growing surface microflora are desirable so that enzymes produced by the surface microflora can diffuse throughout the cheese block. In contrast to surface-ripened cheeses, hard cheeses develop flavor uniformly throughout,

therefore, large blocks of cheese can be manufactured.

Surface-ripened cheeses are ripened in closely controlled conditions of temperature and high relative humidity; 15.6^{o}C and 95% relative humidity are typically used (41, 60). The cheese blocks are placed on wooden shelves inoculated with B. linens. Turning the blocks and brushing them with water or saline solution create a uniform microbial smear on the surface of the cheese. The first stage of ripening may last up to two weeks depending upon the amount of flavor desired in the finished product. After the initial ripening period, the surface-ripened block is wrapped in parchment paper and curing continues for up to eight weeks before the cheese is marketed.

The finished product should be soft bodied with a uniform smear on the outside and a nutty, smooth taste. Overripened cheese will have an aroma and flavor reminiscent of ammonia because of extensive proteolysis, which also yields an overly soft cheese (41).

Summary

Surface-ripened cheeses are those cheeses ripened by a microbial smear growing on the cheese surface. Limburger, Trappist, Leiderkranz, and Brick are examples of this type of cheese. Halophilic yeasts and bacteria are the dominant microorganisms found in the smear. Yeasts appear first upon the cheese and are responsible for creating conditions suitable for bacterial growth. Brevibacterium linens becomes

the dominant bacteria in the smear and is responsible for development of the typical aroma and texture of surface-ripened cheeses. This bacterium may also be responsible for the resistance to molding exhibited by these cheeses.

Aromas of surface-ripened cheeses are complex mixtures. Catabolism of proteins by $\underline{B}$. $\underline{linens}$ is an important source of many of aroma compounds. Sulfur-containing compounds, such as methanethiol, hydrogen sulfide, and thiolesters, are especially important components of surface-ripened cheese aroma. These compounds are known to possess fungicidal activity, and may play a role in antimycotic activity exhibited by surface-ripened cheeses.

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Volatile compounds produced by <u>Brevibacterium</u> <u>linens</u>
inhibit spore germination and growth of
several food-borne molds.

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Running head:

Volatile compounds from <u>B</u>. <u>linens</u> inhibit mold growth.

Volatile compounds produced by <u>Brevibacterium</u> <u>linens</u> inhibit spore germination and growth of several food-borne molds.

Abstract

Volatile compounds produced by <u>Brevibacterium</u> <u>linens</u> ATCC 8377 and 9172 inhibited germination of spores and mycelial growth of <u>Aspergillus</u> <u>parasiticus</u> NRRL 2999, <u>A</u>. <u>clavatus</u> NRRL 6, <u>Penicillium</u> <u>camemberti</u> NRRL 973, and <u>P</u>. <u>expansum</u> NRRL 877. Molds and bacteria were grown on separate agar surfaces in the same enclosed atmosphere using growth chambers constructed of flexible film. A defined medium was used to determine the influence of methionine upon the production of antimycotic, volatile compounds by <u>B</u>. <u>linens</u>. Medium lacking methionine did not inhibit spore germination or hyphal extension.

The presence of <u>Brevibacterium</u> <u>linens</u> in the bacterial smear of surface-ripened cheeses has been correlated with the pungent aroma associated with these cheeses (7, 11, 16). Methanethiol, methyl disulfide, hydrogen sulfide and most recently methylthioacetate have been detected in the aroma of surface-ripened cheeses (7, 8, 17). During ripening of surface-ripened cheeses, amino acids from milk proteins are metabolized by the microflora of the surface smear to form these sulfur-containing compounds. Especially of note is the production of methanethiol (5, 11) and subsequent formation of thioesters (4, 5) by strains of <u>B</u>.

linens.

Volatile compounds produced by bacteria have been
shown to inhibit the growth of molds (9, 10, 15) and mycotoxin
production (2). Ryall et al. (19) demonstrated inhibition of
dermatophytic fungi by brevibacteria grown upon a protein-
aceous medium, composed of synthetic skin and postulated
that methanethiol was the responsible antifungal agent.

B. linens growing upon a cheese generates volatile
compounds, including sulfur-containing compounds (7, 8, 16,
17). This study was undertaken to evaluate the ability of
volatile compounds produced by B. linens to inhibit spore
germination and hyphal growth of molds. The influence of
methionine in the bacterial growth medium was also examined
with regard to germination and hyphal growth.

MATERIALS AND METHODS

Media and organisms. Cultures of Brevibacterium linens ATCC
8377 and 9172 were obtained from the American Type Culture
Collection, Rockville, MD. Stock cultures were maintained
on trypticase yeast extract agar (TYE) which contained (per
liter): trypticase (BBL Microbiology Systems, Cockeysville,
MD), 10 g; yeast extract, 5 g; agar, 15 g (both Difco Labor-
atories, Detroit, MI). B. linens was subcultured bimonthly
onto fresh TYE slants and stored at 4°C.

Aspergillus parasiticus NRRL 2999, Penicillium camem-

berti NRRL 877 and P. expansum NRRL 877 were obtained from
the Northern Regional Research Center, Peoria, IL. A. cla-
vatus NRRL 6 was obtained from the Plant Science Department,
South Dakota State University. All molds were maintained on
malt agar (Difco) supplemented with 3 g yeast extract (MYE)
per liter. Molds were subcultured bimonthly onto fresh MYE
slants and stored at 25 °C.

A defined medium (DMM), which was also used to culture
B. linens, contained buffered salts solution described by
Meynell and Meynell (14); amino acids (125 ug/ml), alanine,
aspartic acid, glutamic acid, lysine, tryptophan, and
methionine (all Sigma Chemical Co., St. Louis, MO); and
vitamins (per liter); biotin, 5 ug, folic acid, 50 ug, and
thiamine, 1.0 mg (all Sigma). Methionine was deleted from
the medium (DMA) for some experiments. When required, media
were solidified with Noble agar (15 g/l). The pH of each
defined medium was adjusted to 7.0.

Spore germination studies. An adaptation of the slide-ger-
mination method of the American Phytopathological Society
(1) was used to evaluate the influence of volatile compounds
produced by strains of B. linens on germination of mold
spores. Suspensions of spores were prepared by rinsing
sporulated cultures with cold, sterile distilled water. The
resulting spore suspension was passed through cheesecloth to
remove mycelial fragments and diluted to contain 5000 to
10,000 spores/ml. Slide cultures were prepared by placing a

drop of molten MYE onto a sterile microscope slide in the
cover of an inverted petri plate and transferring 0.01 ml of
the diluted spore suspension, about 100 spores, onto the
solidified agar.

Bacterial inocula were prepared in a chemically de-
fined medium lacking an organic sulfur source (DMA). Cul-
tures were incubated aerobically at 30 $^{\circ}$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 each
agar surface. Inoculated plates were incubated for 24 h at
30 $^{\circ}$C before preparation of growth chambers.

Growth chambers were prepared by combining covers of
inverted petri plates containing freshly-prepared slide
cultures of spores with the bottoms of petri plates that
contained confluent, 24 h surface growth of B. linens on the
desired agar medium. Combined petri plates were placed in
flexible pouches composed of polyester/polyethylene laminate
(Dazey Corp., Industrial Airport, KS), and the pouches were
heat-sealed to form growth chambers (Fig. 1). Growth cham-
bers were incubated at 25 $^{\circ}$C.

Inhibition of spore germination by volatile sulfur
compounds produced by B. linens was evaluated using trapping
agents to remove specific compounds from the atmospheres of
growth chambers. Aqueous solutions of mercuric chloride, 3%
(w/v), mercuric cyanide, 4% (w/v), and cadmium sulfate 5%
(w/v in 0.2 N HCl) were used as trapping agents (3, 6, 13, 20).

Two leaves of 55 mm filter paper (Whatman No. 1), containing a single trapping solution were used as traps. Traps were placed under the slide culture and only one trapping agent was used in each growth chamber. Distilled water replaced trapping solutions in growth chambers containing no traps and control growth chambers.

Growth chambers were incubated at 25 °C. Control chambers without bacteria and containing slide cultures were opened for evaluation after 24 h. Growth chambers containing B. linens along with slide cultures were opened after 48 h incubation. Spore germination was determined by means of a microscope, a spore was considered germinated only if a germ tube was apparent. Germination of spores in growth chambers was recorded as a percentage of the average number of spores germinating in control growth chambers. Hyphal extension studies. Slide cultures of spores were prepared described for spore germination studies. Mold cultures were incubated for 12-18 h in the absence of bacteria to allow spore germination. As in germination studies, slide cultures were then placed in inverted petri plates containing confluent surface growth of 24-h cultures of B. linens on TYE, DMM, or DMA agar. These combined plates were sealed into growth chambers and incubated at 25 °C. Control chambers contained only slide cultures. Half of the growth chambers containing molds and bacteria were opened after 12 h incubation (Treatment 1), hyphal measure-

ments were made, and slide cultures and bacteria were re-sealed into chambers. This procedure was repeated at 12-h intervals for the duration of the experiment. The remaining growth chambers were incubated for 24 h (Treatment 2), chambers were opened, bacteria were removed, and hyphal measurements were made at 12-h intervals to determine if growth resumed. Hyphal extension measurements were made by means of a microscope fitted with a calibrated ocular micro-meter. Measurements were made at a magnification of 100x and the largest colony diameter was measured. Measurements were made on five randomly selected propagules per chamber, six chambers were used each time, treatment, and medium for a total of 30 measurements.

Statistical interpretation. Spore germination percentages were transformed using $arcsin(\%germination^{0.5})$ and then analysed by analysis of variance, each treatment was repli-cated eight times. Duncan's multiple range test was used to determine differences between treatments. Analysis of covariance was used to interpret hyphal extension data. Significance in all analysis was considered to be at P <

RESULTS AND DISCUSSION

Preliminary studies showed that the trapping agents.

used in this study did not reduce spore germination when B.
linens was absent. Germination of mold spores was inhibited
when spores were incubated in the same atmosphere as B.
linens ATCC 9172 and 8377 (Table 1). A pungent thiol-like
aroma was apparent upon opening growth chambers containing
only bacteria and slide culture (No Trap, Table 1) when TYE
was used as growth medium for B. linens. Spores of all
species of molds tested did not germinate in trapless growth
chambers. Trapping agents included in growth chambers
increased spore germination and reduced the pungent aroma
compared to trapless chambers. Cadmium sulfate traps were
superior to traps containing mercuric chloride or mercuric
cyanide for improving the percentage of spores germinating
compared to trapless chambers. When mercuric cyanide traps
were used in growth chambers, the percentage of spores of A.
parasiticus germinating was increased; however, an increase
in the percentage of spores germinating was not noted for A.
clavatus, P. expansum, or P. camemberti. The percentage of
spores of P. camemberti germinating increased in growth
chambers containing B. linens and mercuric chloride traps.
Spore germination was also improved when P. expansum and B.
linens ATCC 8377 were included in growth chambers containing
mercuric chloride traps. Mercuric chloride traps did not
increase the percentage of spores geminating for the molds
used in the study. Other researchers (6, 11, 12, 13, 20)
have used mercuric cyanide to remove hydrogen sulfide and

thiols, and, mercuric chloride to remove sulfides, disul-
fides, and thiols from atmospheres of several environments.
Cadmium sulfate was used by Walker (20) to remove hydrogen
sulfide from cheese aroma. As used in this study, the
reaction of the trapping agents may have released volatiles
which were inhibitory to spore germination. For example,
reaction of thiols with mercuric cyanide forms hydrogen
cyanide (3). These reaction products may cause the
differential responce of mold spores to the presence of
mercuric chloride or mercuric cyanide and B. linens.

A defined medium lacking organic sulfur, DMA, was
used to determine the influence of methionine upon
production of antimycotic compounds by B. linens (Table 2).
Germination of mold spores was inhibited when methionine was
included in the bacterial growth medium (DMM); however, when
growth chambers contained bacteria grown upon DMA, mold
spore germination was not inhibited. Comparison of spore
germination in chambers containing B. linens grown upon DMA
with spore germination in chambers containing B. linens
grown upon DMM indicates that methionine was necessary for
inhibition.

Extension of mold hyphae was also inhibited by vola-
tile compounds produced by B. linens grown upon TYE or DMM
(Fig. 2-5). Hyphal growth in the presence of B. linens
grown upon DMA (data not shown) was similar to hyphal growth
in control chambers. The bacteria were removed from growth

chambers (Treatment 2, Fig. 2-5) after 24 h to determine if inhibition of hyphal growth was permanent. When treatment two was used, inhibition of hyphal growth was apparent for all molds except P. expansum grown in the presence of B. linens grown upon DMM (Fig. 5). For all other molds, B. linens ATCC 9172 tended to restrict growth of hyphae more than B. linens ATCC 8377 whern the bacteria were grown on DMM. B. linens grown upon TYE inhibited hyphal extension regardless of the treatment or mold. Furthermore, when TYE was used as the bacterial growth medium hyphal lengths were shorter compared to those in growth chambers with B. linens grown upon DMM, albeit inhibition was observed in chambers containing either medium.

Morphological changes of mold hyphae occurred when incubated in the presence of B. linens grown upon TYE or DMM, (Fig. 5). Hyphae became swollen and highly vacuolated after 48 h of incubation in the presence of B. linens (Fig. 5a). In some cases, hyphae appeared to rupture with loss of cytoplasm (Fig. 5b). Hyphae in growth chambers with B. linens grown upon DMA did not exhibit morphological changes (Fig. 5c). Mold spores also exhibited morphological changes when incubated in the presence of B. linens grown upon DMM and TYE. Spores became smaller and appeared to become desicated. Pezet and Pont (18) observed morphogenetic changes in Phomopsis vitacola when the fungi produced methanethiol from a medium containing methionine. Methane-

thiol is also known to cause alterations in the life cycle of _Aphanomyces euteiches_ (12). Methanethiol is known to be produced by B. _linens_ during ripening of surface-ripened cheeses (7, 8, 18).

The pungent aroma noted upon opening growth chambers containing B. _linens_ grown upon TYE or DMM was garlic-like and reminiscent of organic sulfur compounds. Methanethiol, methyl disulfide, and other sulfur-containing compounds are produced by B. _linens_ during aging of surface-ripened cheeses (7, 8, 17). When growth chambers containing B. _linens_ grown upon DMM or DMA were opened only DMM chambers had a pungent aroma. Since the presence of methionine was the only difference between these media it is concluded that B. _linens_ produced a volatile metabolite from methionine that was capable of inhibiting mold growth and spore germination. The ability of B. _linens_ to produce methanethiol from methionine is well-documented (4, 5, 11) and we suggest that this is the compound responsible for inhibition. Furthermore, we suggest that production of volatile compounds such as methanethiol during aging of surface-ripened cheeses is important in suppressing mold spoilage.

Acknowledgements

We are grateful to the Microbiology Department at South Dakota State University for their assistance. J. Bode is to be acknowledged for her dexterity with a microscope.

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TABLE 1. Percentage of mold spores germinating in the presence of <u>Brevibacterium linens</u> ATCC 8377 or 9172 grown upon TYE in the presence of trapping agents.

| Mold | Strain of B. linens | Number of spores in control[a] | Spores germinating, % Trapping Agent | | | No Trap |
			$HgCl_2$	$Hg(CN)_2$	$CdSO_4$	
<u>A. parasiticus</u>	9172	56 ± 1.6	0[c]	92.9 ± 3.7[b]	40.3 ± 6.8[d]	0[c]
	8377	77 ± 2.7	0[c]	83.1 ± 3.5	90.3 ± 4.9	0[c]
<u>A. clavatus</u>	9172	89 ± 5.4	0	0	91.8 ± 6.3[c]	0
	8377	75 ± 2.8	3.3 ± 1.1	2.0 ± 0.7	87.2 ± 3.6[c]	2.7 ± 0.8
<u>P. expansum</u>	9172	109 ± 7.3	0	0	94.1 ± 9.2[c]	0
	8377	88 ± 3.5	94.5 ± 3.1[c]	0	95.6 ± 4.0[c]	0
<u>P. camemberti</u>	9172	74 ± 2.8	83.7 ± 3.6[c]	0	89.2 ± 3.5[c]	0
	8377	95 ± 4.8	81.7 ± 4.2[d]	0	67.9 ± 6.2[c]	0

[a]Average number ± standard error of spores germinating in eight chambers not containing bacteria.

[b]Mean ± standard error of percentage of spores germinating in eight replicates.

[c,d]Values with different superscripts within a row are different (P<.05).

TABLE 2. Percentage of mold spores germinating in the presence of
<u>Brevibacterium</u> <u>linens</u> ATCC 8377 and 9172 grown upon DMA and DMM
media.

| Mold | Strain of B. linens | Spores germinating, % Medium | |
		DMM	DMA
<u>A</u>. <u>parasiticus</u>	9172	0	89.4 ± 4.0[a,b]
	8377	0	99.4 ± 1.3[b]
<u>A</u>. <u>clavatus</u>	9172	0	97.0 ± 5.7[b]
	8377	6.9 ± 1.4	94.2 ± 1.5[b]
<u>P</u>. <u>expansum</u>	9172	0	82.7 ± 7.2
	8377	0	85.7 ± 4.3
<u>P</u>. <u>camemberti</u>	9172	29.2 ± 3.1	90.3 ± 1.1[b]
	8377	0	90.2 ± 2.8[b]

Control = 100% germination.

[a]Mean ± standard error.

[b]Not significantly different from control (P<.05).

Figure 1. Hyphal extension of <u>Aspergillus parasiticus</u> NRRL 2999 in the
presence of <u>Brevibacterium linens</u> ATCC 8377 and 9172 grown upon
DMM (A) or TYE (B). Treatment 1 (+ = 8377, Δ = 9172) - bacteria
remained in growth chambers for the entire experiment. Treat-
ment 2 (□ = 8377, # = 9172) - bacteria removed from growth chambers
after 24 h incubation. Control (X) growth chambers did not con-
tain bacteria.
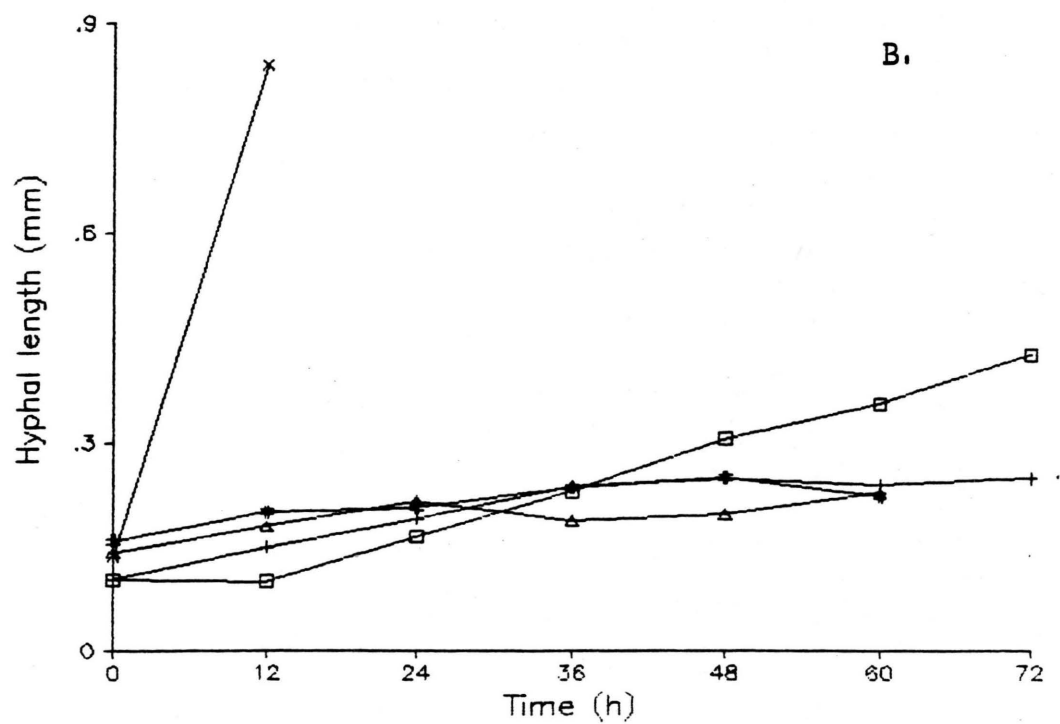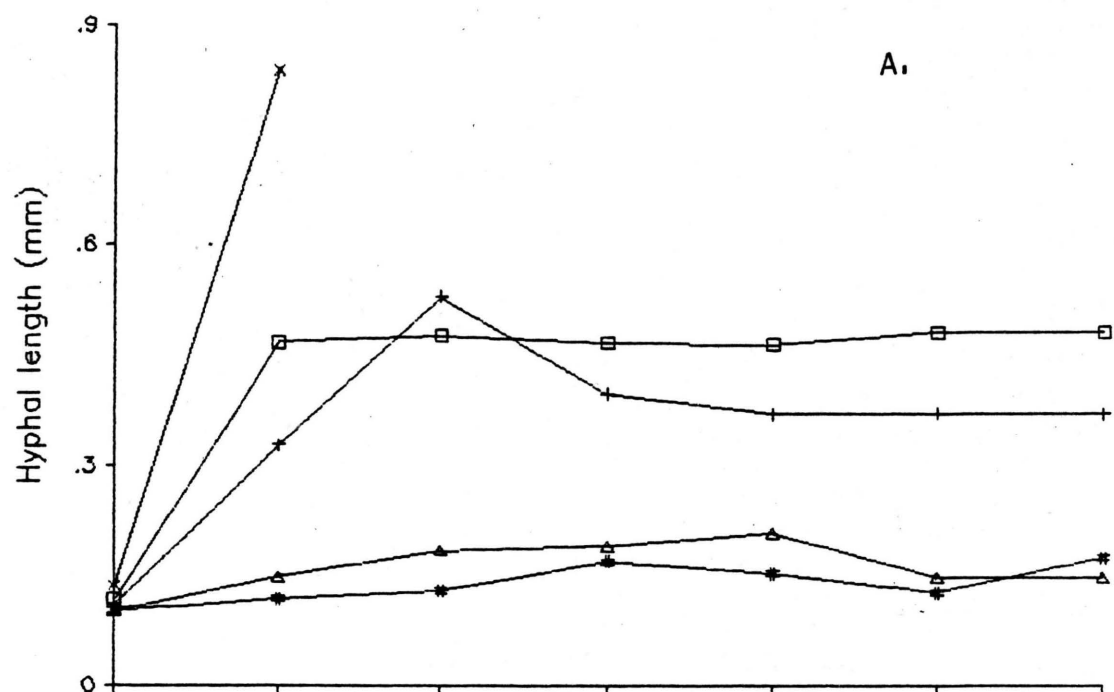
Figure 2. Hyphal extension of <u>Aspergillus</u> <u>clavatus</u> NRRL 6 in the presence of <u>Brevibacterium</u> <u>linens</u> ATCC 8377 and 9172 grown upon DMM (A) and TYE (B). Treatment 1 (+ = 8377, $\triangle$ = 9172) - bacteria remained in growth chambers for the entire experiment. Treatment 2 ($\square$ = 8377, # = 9172) - bacteria removed from growth chambers after 24 h incubation. Control (X) growth chambers did not contain bacteria.
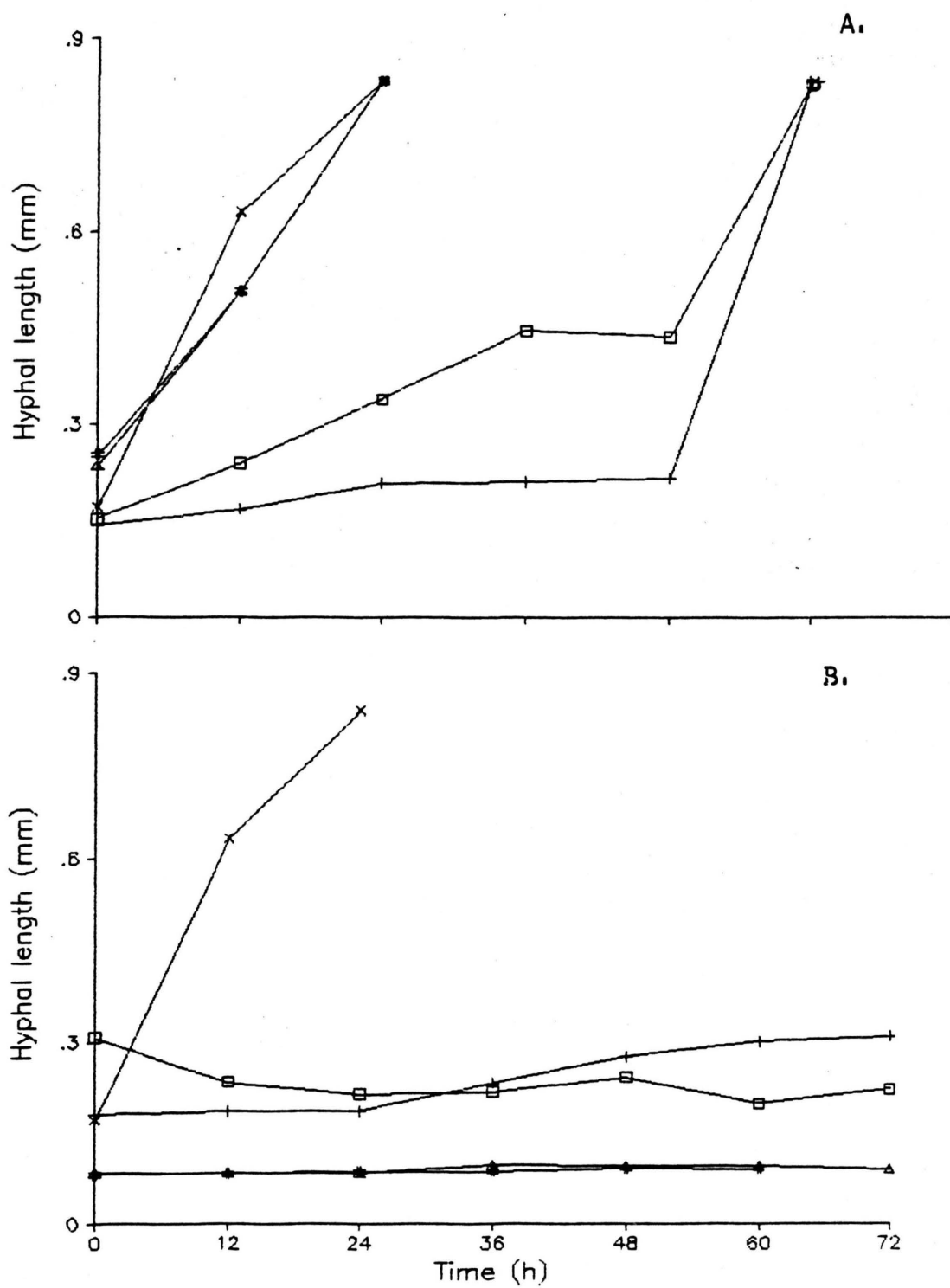
Figure 3. Hyphal extension of <u>Penicillium camemberti</u> NRLL 973 in the
presence of <u>Brevibacterium linens</u> ATCC 8377 or 9172 grown
upon DMM (A) or TYE (B). Treatment 1 (+ = 8377, △ = 9172) -
bacteria remained in growth chambers for the entire experi-
ment. Treatment 2 (□ = 8377, # = 9172) - bacteria removed
form growth chambers after 24 h incubation. Control (X)
growth chambers did not contain bacteria.
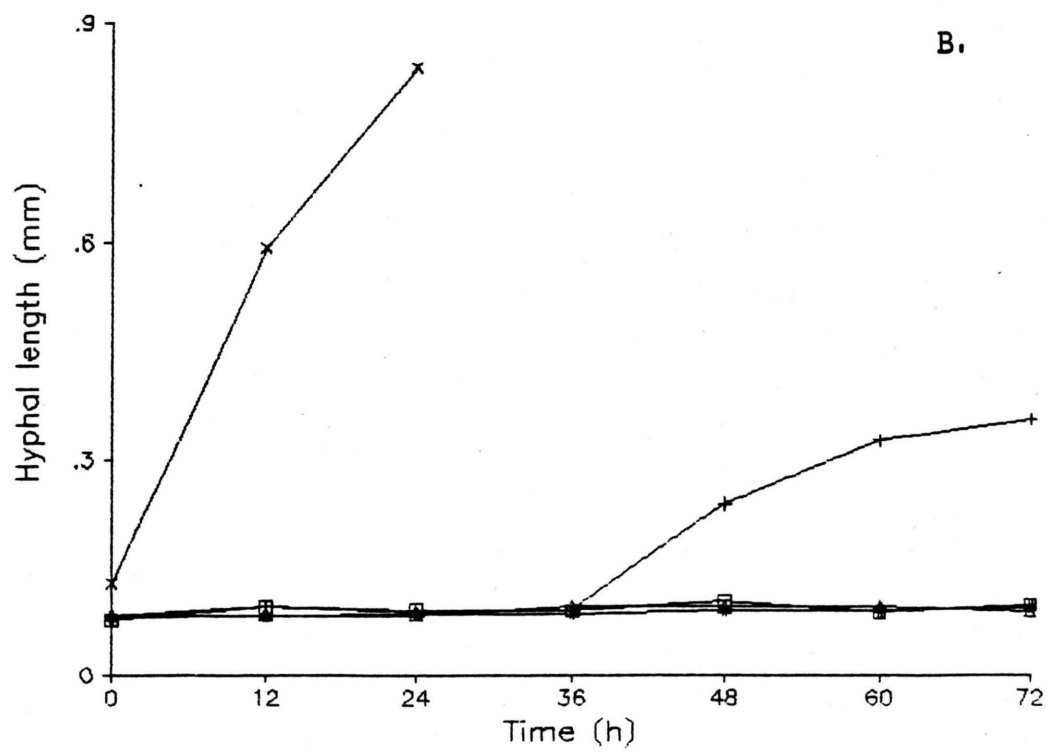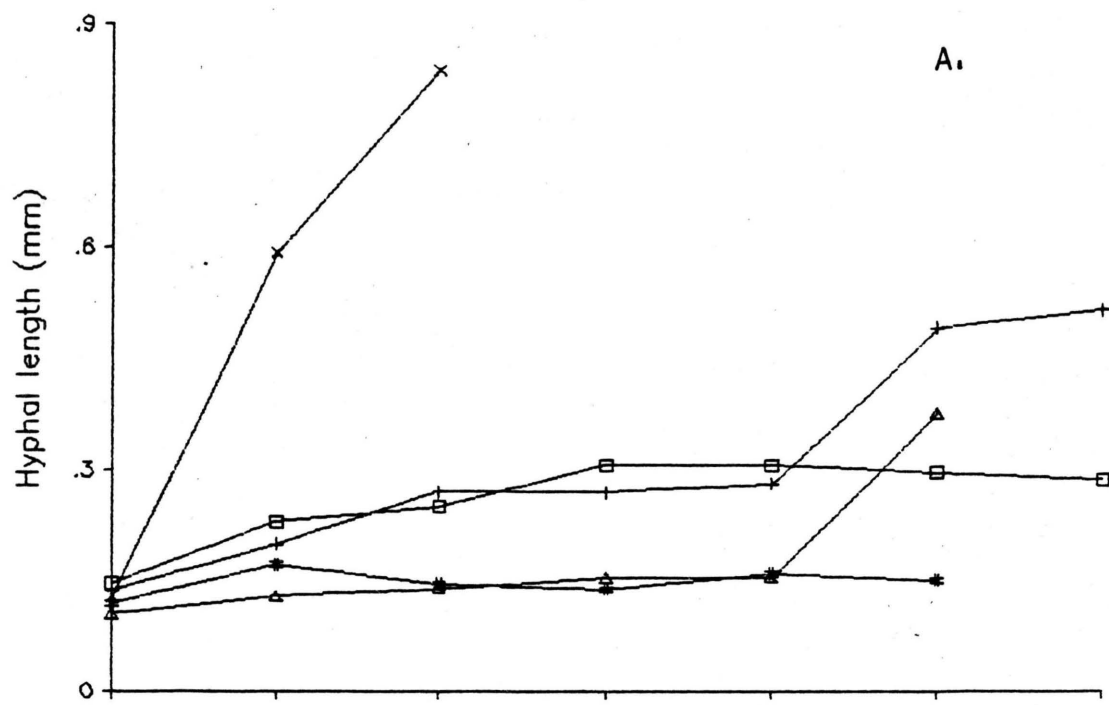
Figure 4. Hyphal extension of Penicillium expansum NRRL 877 in the
           presence of Brevibacterium linens ATCC 8377 and 9172 grown
           upon DMM (A) or TYE (B). Treatment 1 (+ = 8377, Δ = 9172) -
           bacteria remained in growth chambers for the entire experi-
           ment. Treatment 2 (□ = 8377, # = 9172) - bacteria removed
           from growth chambers after 24 h incubation. Control (X)
           growth chambers did not contain bacteria.
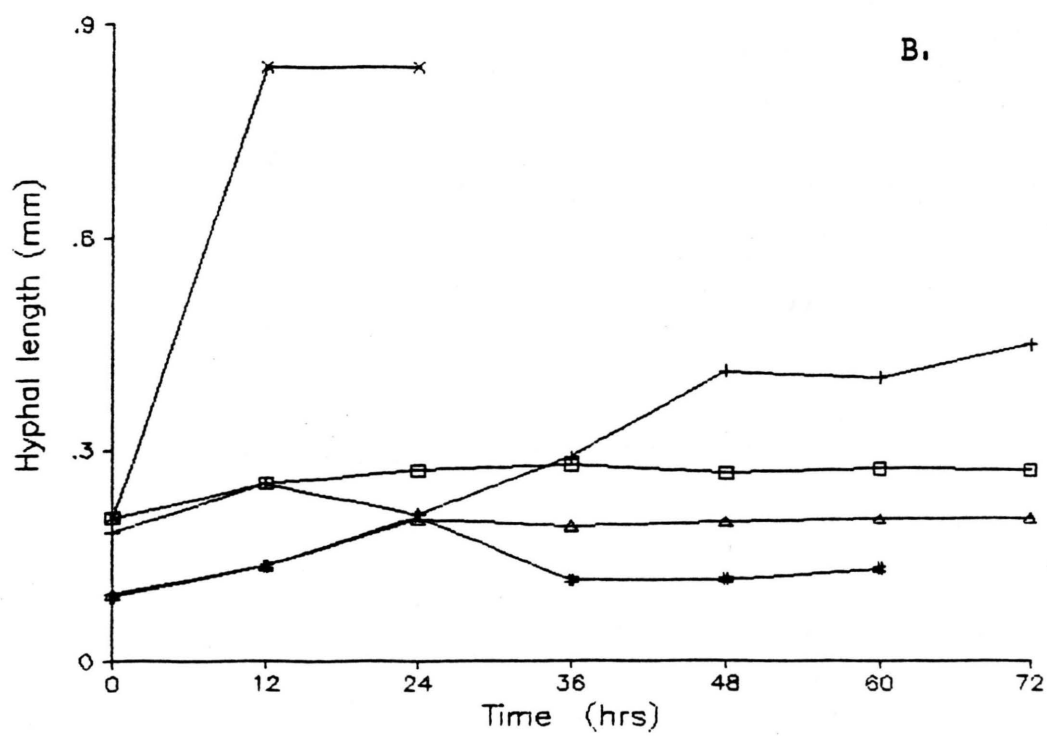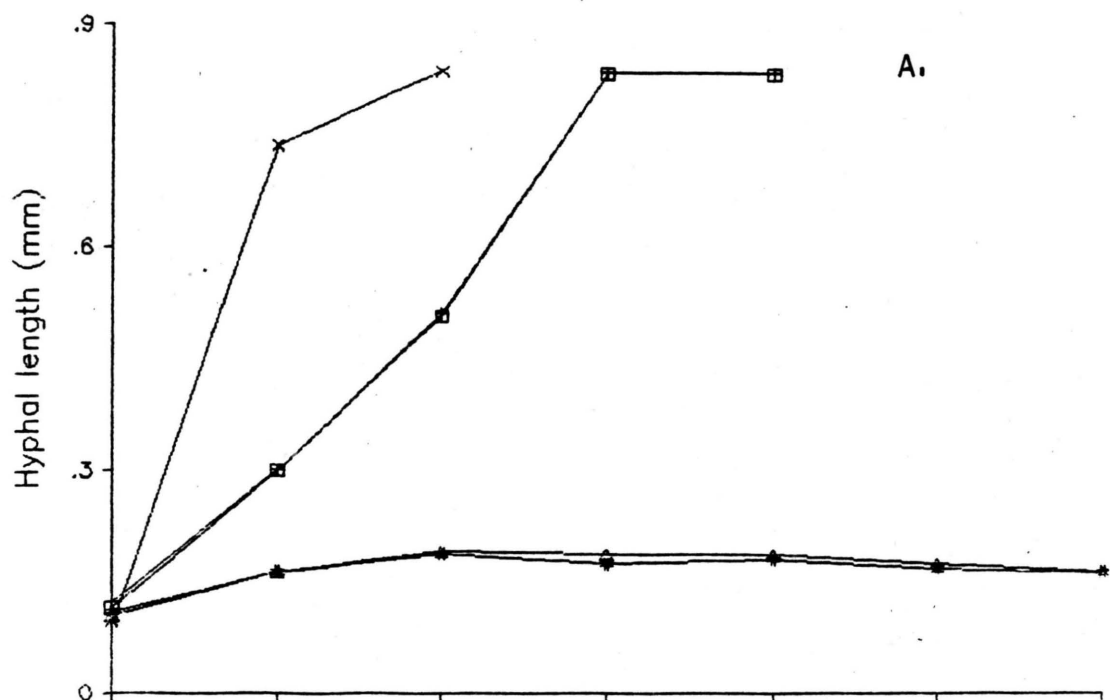
Figure 5. Micrographs showing morphological changes in hyphae
of molds caused by volatile compounds produced by
Brevibacterium linens ATCC 8377 grown upon DMM (400 x).
A - Aspergillus parasiticus, B - Penicillium camemberti,
C - Aspergillus parasiticus control.
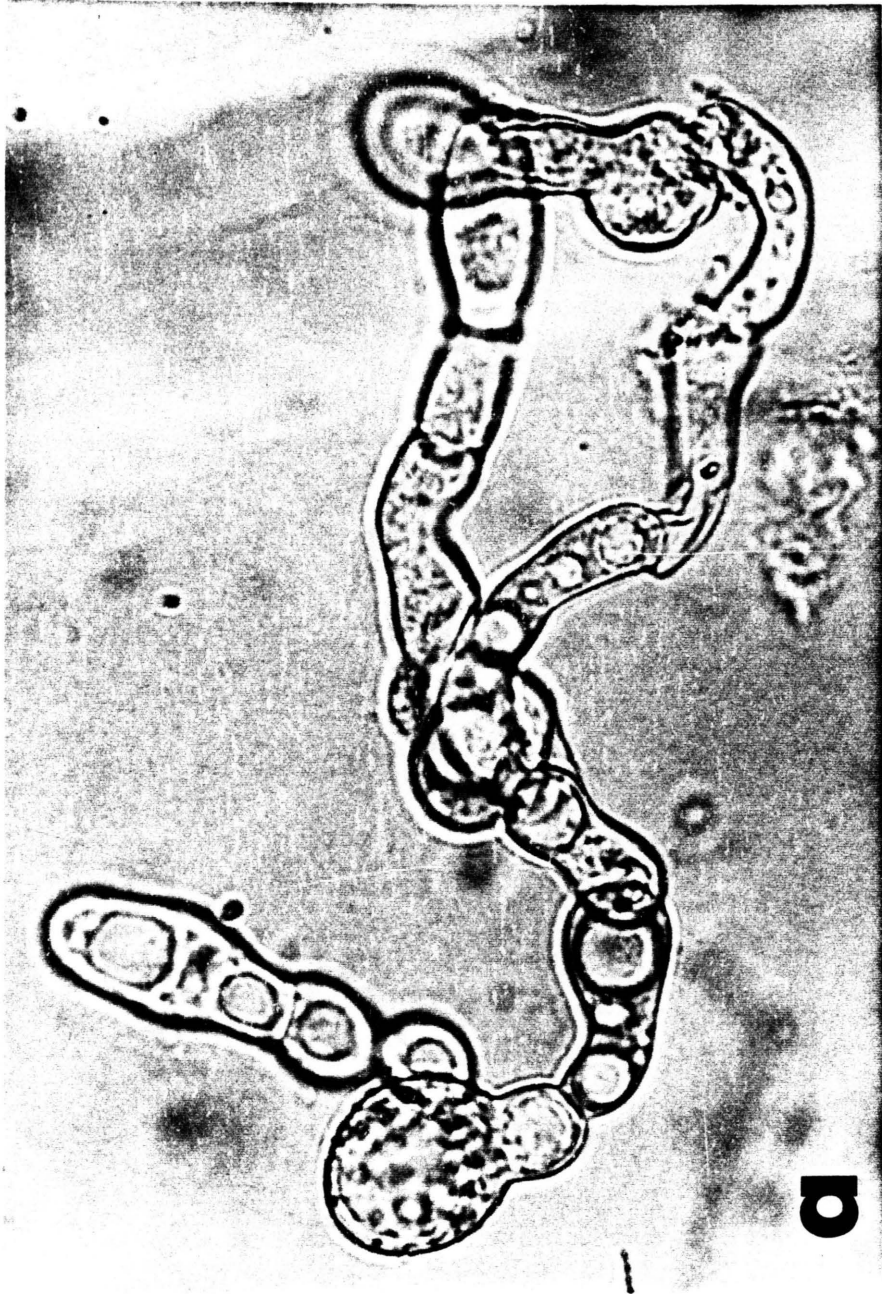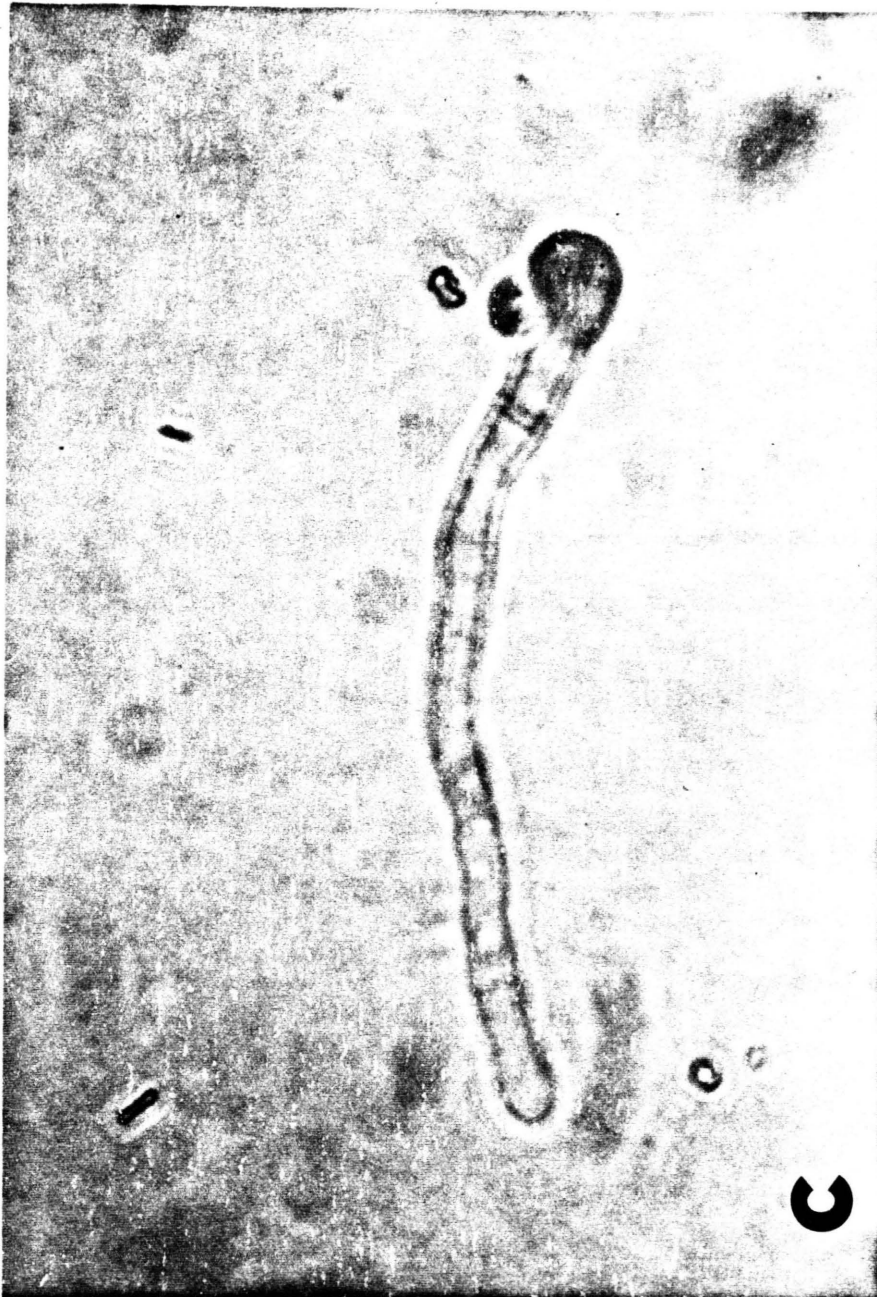
Fungal toxicity of volatile compounds produced by

Brevibacterium _linens_ caused by methanethiol

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Abstract

Methanethiol production was studied using 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 are 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., 1983). Grill et al. (1967) found that the surface smear of Trappist cheese was necessary for production of methanethiol from methionine. Methanethiol, hydrogen sulfide, and methylthioacetate 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 possiblity that an antibiotic, produced by B. linens, was responsible for the resistance to molding. 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 irreversibly spore germination and mycelial growth of several mold species commonly reported on spoiled cheese (Beattie and Torrey, 1985), and inhibition of mold growth depended upon the presence of methionine in bacterial growth media. We report herein on the toxicity of methanethiol on spores of Penicillium expansum, the rate of production of methanethiol by B. linens, and use of polyester/polyethylene laminate pouches as controlled atmosphere growth chambers.

MATERIALS AND METHODS

Organisms and culture conditions. Brevibacterium linens
ATCC 8377 was obtained from the American Type Culture Col-
lection, Rockville, MD, and Penicillium expansum NRRL 973
was obtained from the Northern Regional Research Center,
Peoria, IL. Malt agar (Difco Laboratories, Detroit, MI)
supplemented with 3 g/L yeast extract (MYE) was used for
growth and maintenance of P. expansum. A medium (TYE)
containing trypticase (BBL Microbiology Systems, Cockeys-
ville, MD), 10 g/L; yeast extract, 5 g/L; and agar, 15 g/L
(both Difco) was used for growth and maintenance of B.
linens.

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

Bacterial inocula were prepared in DMA broth; these
cultures were incubated aerobically at 30 $^{\circ}$C for 12-18 h.
Petri plates containing TYE, DMM, or DMA agar were inocu-

lated 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 x 24.4 cm), composed of polyester (0.5 mil)/polyethylene (1.5 mil) laminate (Dazey Corp., Industrial Airport, KS). 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 of growth chambers were taken through the septum by means of a gas-tight syringe. Growth chambers were incubated at 25 ^{o}C.

Analyses of growth chamber atmospheres. Atmospheres samples were directly injected 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 x 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 ^{o}C, and the oven was programmed (without delay) from 40 to 110 ^{o}C at 8 ^{o}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 .71 min, 3.36 min, and 2.27 min, respectively.

Stock mixtures of methanethiol in nitrogen gas were made in empty sealed pouches. Evacuated pouches were rein-flated 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 stan-dard curve correlating peak height 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 $\underline{P}$. $\underline{expansum}$. A drop of MYE on a sterile microscope slide was inoculated with 50 to 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. Atmo-spheres 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.

Growth chambers were incubated at 25 ^{o}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). Spores did germinate in chambers containing B. linens grown upon 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 atmospheres 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 which lacked methionine. However, when sam-
ples from chambers containing B. linens grown on media
including protein (TYE) or methionine (DMM) were chromato-
graphed (Figure 1), methanethiol and methyl disulfide were
detected but not methylthioacetate. Methyl disulfide, pro-
bably 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 1), 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.

Concentrations of methanethiol in growth chambers
were determined at intervals during incubation (Figure 2).
Data from growth studies in this laboratory demonstrated
that toxic concentrations of methanethiol occur in growth
chambers after 24 to 36 h incubation on DMM and TYE media.
Examination of Figure 2 reveals that methanethiol concen-
trations on both media during this period ranged from about
0.2 to 1.2 ppm.

Spores of P. expansum were used to correlate concen-
tration of methanethiol with toxicity. A series of growth
chambers containing slide cultures of spores were injected
with varying concentrations of methanethiol, and the cham-

bers then incubated. Spore germination was inhibited permanently by the presence of 0.82 but not 0.33 or 0.16 ppm methanethiol (Table 1).

Cuer et al. (1979) have reported methanethiol, hydrogen sulfide, methyl disulfide, and methylthioacetate in volatiles from strains of B. linens cultured on a medium containing protein. Methanethiol produced during decay of cruciferous plants inhibited fungal 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. We report herein the capability of methanethiol in concentrations less than 1 ppm to inhibit germination of spores of P. expansum. B. linens grown on DMM or TYE produced methanethiol in concentrations similar to those found toxic to the fungi. Production of methanethiol by B. linens during the manufacture of surface-ripened cheeses serves to discourage mold spoilage.

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Figure 1. Diagram of 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.
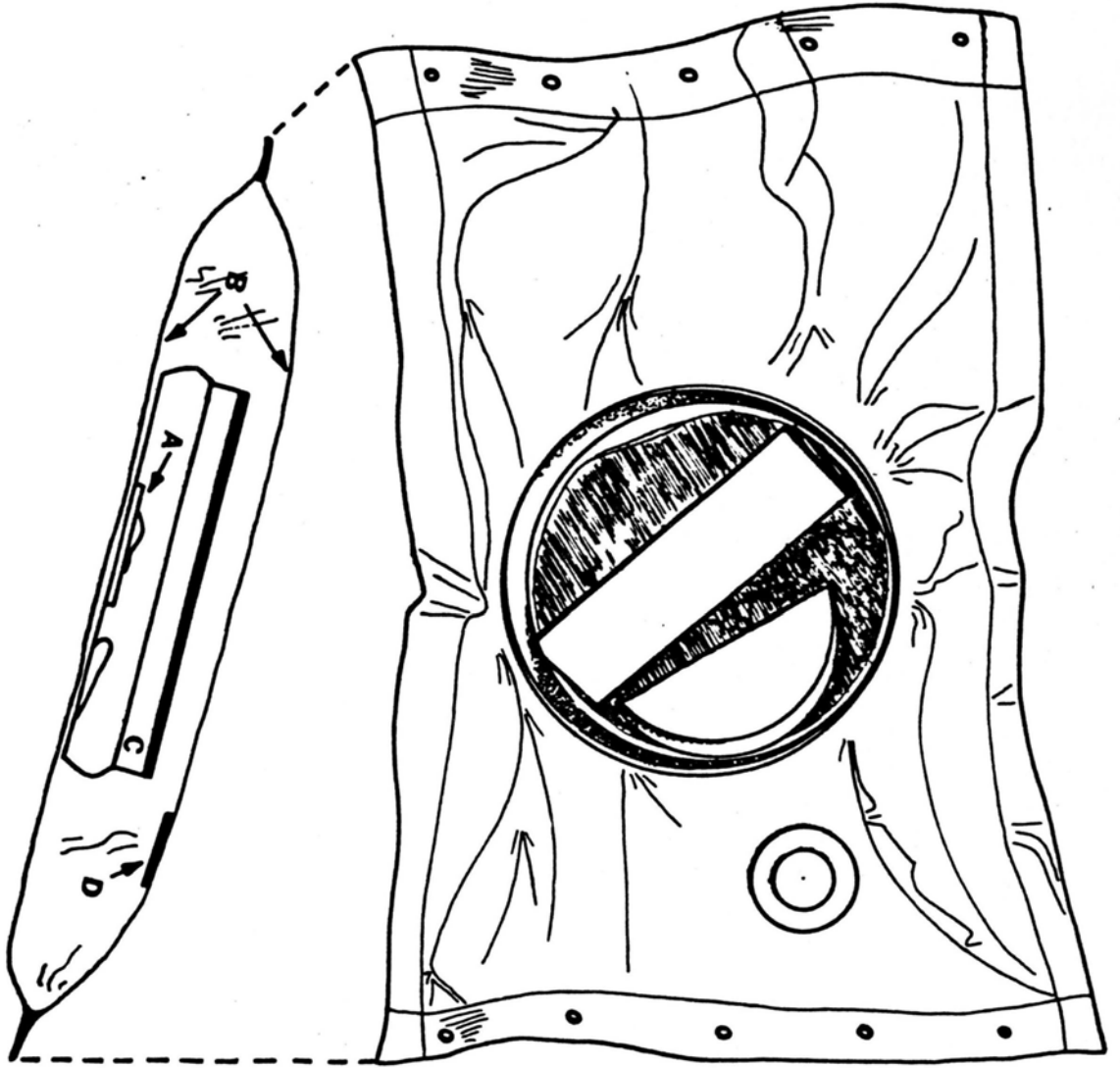
Figure 2. Chromatogram of atmosphere from growth chamber containing
Brevibacterium linens ATCC 8377 grown upon DMM medium for
84 h. Attenuation 32x at 10^{-12} sensitivity. A. Methane-
thiol. B. Methyl disulfide.

Time (min)
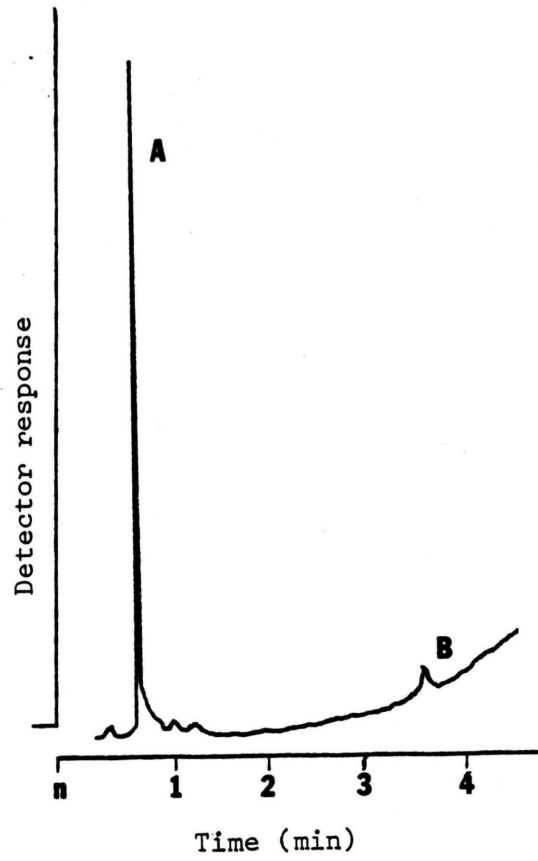
Figure 3. Methanethiol production by <u>Brevibacterium</u> <u>linens</u> ATCC 8377 grown upon DMM (●) and TYE (■) media, average of three replications.
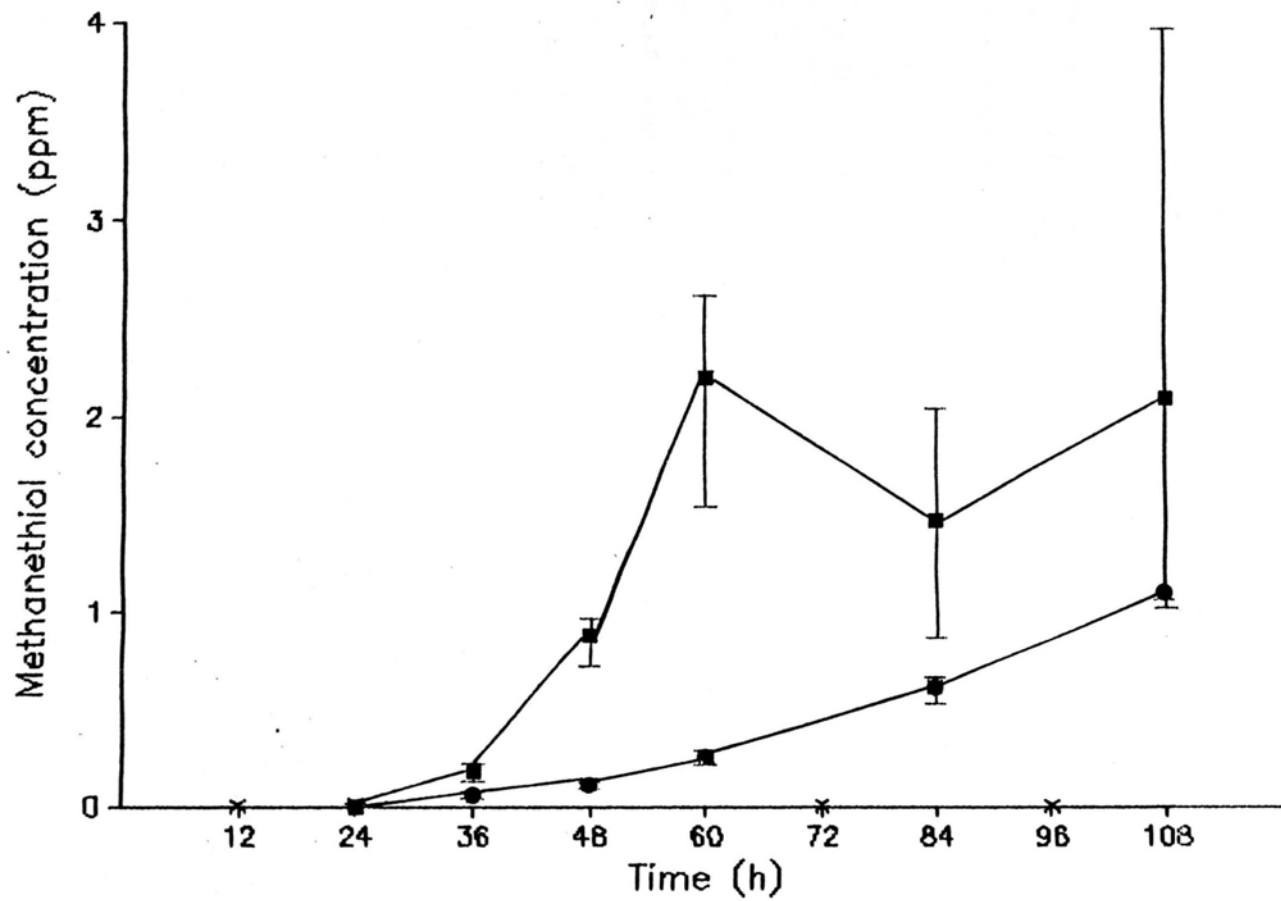
Table 1. Effect of methanethiol concentration on germination of spores of <u>Penicillium</u> <u>expansum</u> NRRL 973.

Methanethiol concentration	Number of spores germinated
0.0[a]	107 ± 5 [b]
0.16	107 ± 6
0.33	86 ± 46
0.82	0
1.6	0
3.3	0

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

SUMMARY OF CONCLUSIONS

Volatile compounds produced by Brevibacterium linens inhibited spore germination and hyphal growth of several molds. Inhibition of molds inside growth chambers in which molds and bacteria were grown on separate media was not observed when methionine was excluded from bacterial media. Incorporation of methionine in bacterial media caused inhibition of mold growth and resulted in production of methanethiol by B. linens. Methanethiol, in concentrations similar to those produced by B. linens in growth chambers was shown to inhibit germination of mold spores. Production of methanethiol in surface-ripened cheese may be responsible, in part, for the resistance to molding these cheese exhibit.

Appendix A

Development of defined medium

The ability of a medium to support bacterial growth can be determined by measuring an increase in cell concentration after a period of growth. The increase in biomass over a period of time can be described mathematically as $dx/dt = ux$ (2, 9); where dx/dt is change in cell concentration, dx, over time interval, dt. x is the actual cell concentration at any given time, and, u is the specific growth rate (h^{-1}). This formula is valid only during the exponential phase of growth, and for a given microorganism in a given medium under constant conditions, u is a constant and the slope of the exponential portion of a growth curve.

In dilute cultures, cell concentration and medium turbidity are directly related (2), therefore, the amount of light absorbed or transmitted through a liquid culture can be related to bacterial growth and actual cell concentration. The slope of the growth curve during exponential growth in a dilute culture is an indication of the ability of a medium to support bacterial growth.

Growth of Brevibacterium linens upon a complex medium such as cheese yields a variety of compounds, most notable are sulfur compounds which are important components of cheese aroma (1, 4). A defined medium was developed to determine the influence of organic sulfur upon the production of volatile sulfur compounds by B. linens. Growth curves were done to determine the influence of an added organic sulfur source upon the growth of B. linens.

Materials and Methods

The media used for growth curves are given in Figure A1. Stock cultures of B. linens ATCC 8377 and 9172, obtained from the American Type Culture Collection, were streaked onto a medium (TRP) containing; trypticase, 1% (BBL Microbiology Systems, Cockeysville, Md), and agar, 1.5% (Difco Laboratories, Detroit, MI). Growth from an isolated colony on TRP was used to inoculate 25 ml of test medium. Flasks of inoculated test medium were placed upon an incubator-shaker (New Brunswick Scientific, Edison, NJ) and incubated at 30 ^{o}C for 24 h with agitation (200 rpm). If a test medium did not become turbid after 24 h incubation it was discarded. Turbid cultures were gram stained to insure purity. These cultures were used as inocula for growth curves.

Growth curves were initiated by inoculating 50 ml of the test medium in a 300 ml side-arm flask. A 3% inoculum was used for all media except those containing 5 or 6 amino acids, these were inoculated at 10%. A spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY) was used to determine initial (time = 0) absorbance of media. Cuvettes containing uninoculated media were used as blanks. Inoculated side-arm flasks were incubated as described earlier and absorbance readings were made periodically.

Results and Discussion

The goal of this research was to develop a chemically defined medium that would support growth of B. linens in the absence of an organic sulfur source. This medium, as developed or supplemented with methionine, was used to evaluate the production of antimycotic volatiles by B. linens. The medium selected contained five amino acids, three vitamins, and basal salts (DMA, Figure A1). When an organic sulfur source, methionine, (DMM) was added, growth of B. linens was not decreased (Figure A2). Specific growth rate constants of B. linens grown upon DMA and DMM were quite similar (Table A1). While growth of B. linens on DMA or DMM, as evidenced by smaller values for u (Table A1), was slower than on other media, the cell morphology remained constant. There is a tendency for B. linens and other corynebacteria to to become pleomorphic when media is nutritionally inadequate (10, 11).

Figure A1. Media used in development of a defined medium
for the growth of B. linens ATCC 8377 and 9172.

Trypticase yeast extract (TYE):   trypticase, 0.5%; yeast

       extract, 0.5%.

1% Trypticase (TRP):   trypticase, 1%.

20 amino acids (20 AA):

    Amino acids (125 ug/ml): cystine, tryptophan, aspartic

        acid, tyrosine, glutamic acid, arginine, proline,

        asparagine, threonine, isoleucine, methionine,

        phenylalanine, serine, glycine, alanine, valine,

        lysine, leucine, histidine, cysteine.

    Vitamins: biotin, 0.005 ug/ml; folic acid, 0.05 ug/ml;

        thiamine, 1.0 ug/ml.

    Basal salts solution (trace quantities):   NaCl, KCl,

        NH_4Cl, $MgCl_2$, KH_2PO_4, Na_2PO_4, $FeCl_3$ in Tris
        buffer, pH 7.0 (8).

11 amino acids (11 AA):

    Amino acids (125 ug/ml):   tyrosine, lysine, tryptophan,

        methionine, cysteine, proline, alanine, threonine,

        glutamic acid, aspartic acid, isoleucine.

    Vitamins: same as 20 AA medium.

    Basal salts solution: same as 20 AA medium.

Continues,

Figure 1A. (continued)

6 amino acids (DMA^a, DMM):

 Amino acids (125 ug/ml): lysine, alanine, glutamic

   acid, aspartic acid, tryptophan, methionine.

 Vitamins and basal salts solution same as 20 AA medium.

Media brought to neutrality (pH 7.0) by addition of 0.1 N
  NaOH or HCl.

^{a} Methionine not added to DMA medium.

Table Al.   Specific growth rate constants of B. linens ATCC
8377 and 9172 grown at 30 oC upon various media.

Medium	Strain of B. linens	Growth constant, u* (h-1
TRP	8377	.134
	9172	.127
TYE	8377	.115
	9172	.168
20 AA	8377	.113
	9172	.157
11 AA	8377	.153
	9172	.154
DMM	8377	.041
	9172	.043
DMA	8377	.039
	9172	.038

---

*
Growth constant derived from regression equation of the
exponential portion of growth curve, f(x) = ux + b (2, 9).

Figure A2. Growth of <u>Brevibacterium</u> <u>linens</u> ATCC 9172 in six different media at 30°C. □ - TRP, ■ - TYE, ▽ - 20 aa, ● - 11 aa, ▲ - DMA, ○ - DMM.
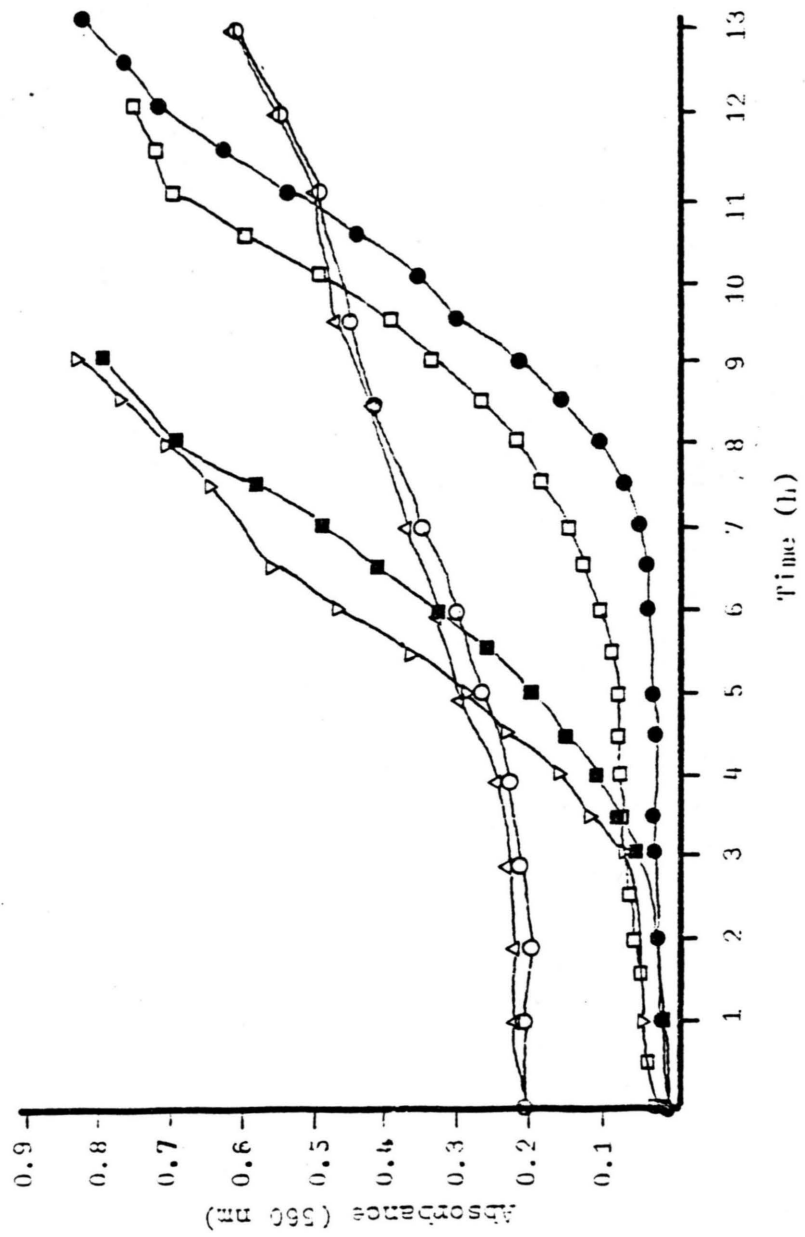
Figure A2. Growth of <u>Brevibacterium</u> <u>linens</u> ATCC 8377 in six different media at 30°C. □ - TRP, ■ - TYE, ▽ - 20 aa, ● - 11 aa, △ - DMA, ○ - DMM.

Absorbance (560 nm) vs Time (h)
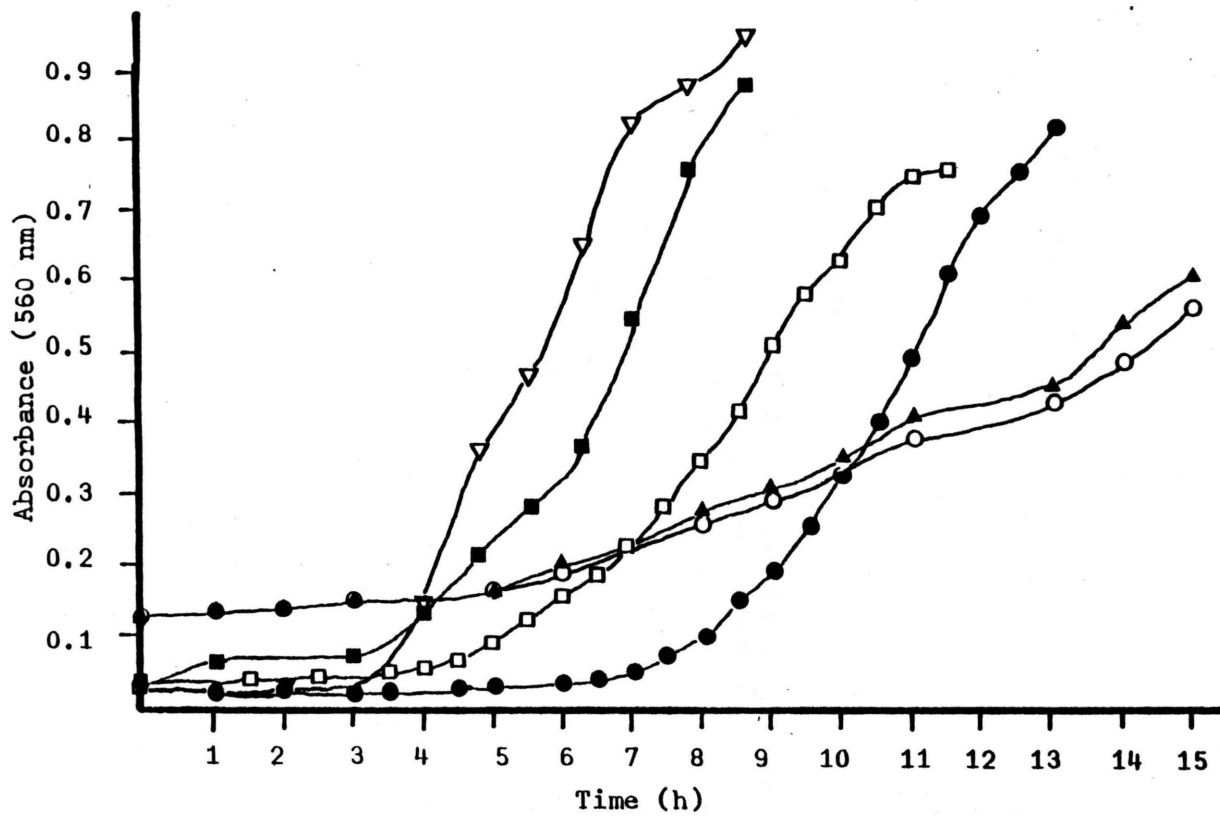
Appendix B

Chemical methods: quantitation of volatile fatty acids,
and efficiency of chemical traps for certain
sulfur compounds

Gas chromatography was done to determine if volatile fatty acids (VFA) were present in atmospheres of growth chambers and in broth cultures of B̲r̲e̲v̲i̲b̲a̲c̲t̲e̲r̲i̲u̲m̲ l̲i̲n̲e̲n̲s̲. Chemical trapping agents were used in growth chambers to remove specific sulfur-containing compounds (Table B1) from the atmospheres of growth chambers. The relative efficiency of these trapping agents in removing the specific sulfur-containing compounds was also determined by gas chromatography.

M̲a̲t̲e̲r̲i̲a̲l̲s̲ a̲n̲d̲ m̲e̲t̲h̲o̲d̲s̲

The chromatograph used was a Varian 1400 equipped with a flame-ionization detector and a stainless steel column (1.83 m x 0.32 cm) packed with 10% SP-1200, 1% H_3PO_4 on Chromosorb W(AW) 80/100 mesh (Supelco, Inc., Bellefonte, PA). The gas chromatograph was operated isothermally at $140\ ^{\circ}C$.

Samples of growth chamber atmospheres and broth culture media were analysed for VFA. Broth cultures of B̲. l̲i̲n̲e̲n̲s̲ were incubated for 24 h on an incubator/shaker and incubated at $30\ ^{\circ}C$ for 24 h with agitation (200 rpm). Following incubation, cultures were acidified with 25% meta-phosphoric acid (2 ml HPO_3 /10 ml media) and centrifuged at 10,000 x g for 10 min to remove bacterial cells from culture media (12). A portion of the supernatant was injected into the chromatograph. Growth chamber atmospheres were sampled by means of a gas-tight syringe and the samples were

injected directly into the chromatograph.

Retention times of peaks occurring in sample chromatograms were compared with retention times of peaks occurring in chromatograms of standards. The VFA standard solution contained the acids: acetic, 3.15 mg/ml; propionic, 0.993 mg/ml; butyric, 0.958 mg/ml; valeric, 0.47 mg/ml; and iso-valeric, 0.431 mg/ml. Standards were run at chromatograph settings of 10^{-11} sensitivity and 128x attenuation, samples were run at 10^{-12} sensitivity and 32x attenuation.

Atmospheres of growth chambers containing B. linens and trapping agents were analysed by means of gas chromatography. A Varian 3700 gas chromatograph equipped with a flame-ionization detector and glass column (1.83 m x 0.63 cm) packed as described by Leppanen et al. (5) was used. The chromatograph was temperature programmed from 40 to 110 ^{o}C at 8 ^{o}C/min. Samples of atmospheres were injected directly into the gas chromatograph.

Results and Discussion

Volatile fatty acids were not detected in the atmospheres of growth chambers, very small amounts of acetic, propionic, and isovaleric acids were found in supernatents of broth cultures. Hosono et al. (as cited in 1) found that B. linens produces organic acids when grown upon casein. Further work by Hosono et al. (as cited in 1) determined that acetic and isovaleric acids originate from alanine and leucine, respectively.

Since no VFA were detected in samples of atmospheres of growth chambers, it is reasonable to conclude that these compounds do not play a role in the observed fungitoxicity of volatile compounds produced by B. linens. Volatile fatty acids found in broth cultures most likely remain in the salt form until acidification of the growth medium by metaphosporic acid.

The trapping agents used in this study (Figure B1) have been used by others (3, 4, 6, 7, 15) to study the effect of removing specific sulfur-containing compounds from cheese aroma and from atmospheres above decomposing cruciferous plants (6). Manning and Price (7) determined that traps composed of mercuric cyanide and mercuric chloride were up to 99% effective in trapping the sulfur compounds for which they were intended. Cadmium sulfate was used by Walker (15) to remove hydrogen sulfide in the presence of thiols and water vapor.

Gas chromatography of atmospheres of growth chambers containing trapping compounds and B. linens revealed that mercuric chloride and mercuric cyanide were very effective in trapping methanethiol. After incubation of B. linens in the presence of these trapping agents for 48 h, methanethiol was not detected in samples from growth chamber atmospheres. The aroma emitted from growth chambers was also changed, the putrid, sulfury aroma apparent in chambers containing only B. linens was completely eliminated by including traps of

mercuric cyanide or mercuric chloride in growth chambers.
Chambers containing traps of cadmium sulfate were less
effective in removing the typical aroma, and, gas chromato-
graphy of atmospheres revealed small concentrations of
methanethiol, about 0.16 ppm.

Table B1. Chemical traps employed to remove sulfur-containing compounds from atmospheres in growth chambers.

Trap	Concentration of trapping agent	Compounds trapped
Mercuric chloride	3% (w/v)	Thiols, sulfides, disulfides
Mercuric cyanide	4% (w/v)	Thiols, hydrogen sulfide
Cadmium sulfate	5% (w/v)	Hydrogen sulfide

Appendix C

Statistics and summary of raw data

Hyphal extension data for all molds with both strains of B̲r̲e̲v̲i̲b̲a̲c̲t̲e̲r̲i̲u̲m̲ l̲i̲n̲e̲n̲s̲ have been summarized and mean values with standard deviations are given in Tables 1 to 8. Each value in these tables is the average from thirty measurements, five measurements taken from six slide cultures for each treatment. There were three treatments for each set of mold and bacterium, however, only treatments one and two are given in the following tables. Treatment three was a control containing no bacteria. Hyphal extension of molds in treatment three chambers proceeded at the same rate as hyphal extension in chambers containing B̲. i̲n̲e̲n̲s̲ grown upon DMA. Treatment one growth chambers contained B̲. l̲i̲n̲e̲n̲s̲ and mold hyphae for the entire experiment. Treatment two growth chambers contained B̲. l̲i̲n̲e̲n̲s̲ for only 24 hours after this period of incubation the slide culture was re-moved from the growth chamber and sealed into a chamber without the bacterium.

The remaining tables are summararies of statistics from Statistical Analysis System (SAS, 13) output. Hyphal extension data was evaluated using general linear model (GLM) procedure and deleting treatment three and DMA data. Spore germination data was evaluated using analysis of variance (ANOVA), differences between means of treatments of spore germination data were determined by Duncans multiple-range test with significance set at 0.05 level (13, 14).

Table C1. Aspergillus parasiticus hyphal extension in the presence of B. linens ATCC 8377 grown upon DMM, DMA, or TYE.

Time (h)	Medium											
	DMA				DMM				TYE			
	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd
0	.09[a]	.03	.10	.03	.10	.03	.10	.03	.14	.06	.16	.05
12	.84	---	.84	---	b		.12	.04	b		.20	.07
24	.84	---	.84	---	.19	.06	.13	.04	.22	.10	.21	.09
36					.19	.06	.17	.06	.19	.09	.23	.12
48					.21	.07	.16	.05	.20	.09	.25	.13
60					.15	.05	.13	.05	.23	.10	.22	.10
72					.15	.06	.18	.06	.22	.06	.25	.11

[a] Mean and standard deviation of 30 measurements, in millimeters, taken from six microscope slide cultures, five measurements/slide culture.

b - Measurement not made because of experimental design, treatment 2 contained bacteria and mold in constant contact for 24 h.

Table C2. Aspergillus parasiticus hyphal extension in the presence of B. linens ATCC 9172 grown upon DMM, DMA, or TYE.

| | Medium | | | | | | | | | | | |
| | DMA | | | | DMM | | | | TYE | | | |
Time (h)	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd
0	.14[a]	.04	.12	.04	.11	.05	.12	.03	.10	.03	.10	.03
12	b		.82	.08	b		.47	.15	b		.10	.02
24	.84	---	.84	---	.53	.16	.48	.13	.19	.10	.16	.08
36	.84	---	.84	---	.40	.15	.47	.15	.24	.14	.23	.12
48					.37	.15	.47	.14	.25	.15	.31	.14
60					.37	.16	.48	.15	.24	.13	.35	.14
72					.37	.15	.48	.16	.25	.13	.42	.25

a
    Mean and standard deviation (Sd) of 30 measurements, in millimeters, taken from six microscope slide cultures, five measurements/slide culture.

    b - Measurement not made because of experimental design, treatment 2 contained bacteria and mold in constant contact for 24 h.

Table C3. Aspergillus clavatus hyphal extension in the presence of B. linens ATCC 9172 grown upon DMM, DMA, or TYE.

	Medium											
	DMA				DMM				TYE			
Time (h)	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd
0	.11a	.03	.10	.03	.12	.04	.12	.05	.18	.07	.20	.06
12	b		.53	.13	b		.30	.07	b		.25	.08
24	.84	---	.84	---	.51	.10	.51	.11	.21	.08	.27	.07
36	.84	---	.84	---	.84	---	.84	---	.29	.05	.28	.07
48					.84	---	.84	---	.41	.03	.27	.07
60									.40	.05	.27	.06
72									.45	.03	.27	.08

a
Mean and standard deviation (Sd) of 30 measurements, in millimeters, taken from six micro-scope slide cultures, five measurements/slide culture.

b - Measurement not made because of experimental design, treatment 2 contained bacteria and mold in constant contact for 24 h.

Table C4. <u>Aspergillus</u> <u>clavatus</u> hyphal extension in the presence of <u>B</u>. <u>linens</u> ATCC 8377 grown upon DMM, DMA, or TYE.

| | Medium | | | | | | | | | | | |
| | DMA | | | | DMM | | | | TYE | | | |
Time (h)	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd
0	.14a	.04	.12	.04	.10	.03	.11	.02	.09	.04	.09	.03
12	b		.84	---	b		.16	.04	b		.13	.04
24	.84	---	.84	---	.19	.05	.19	.05	.20	.07	.20	.09
36	.84	---	.84	---	.19	.03	.18	.04	.19	.06	.11	.05
48					.19	.04	.18	.04	.20	.06	.12	.05
60					.18	.04	.17	.04	.20	.04	.13	.05
72					.17	.04	.16	.04				

a
    Mean and standard deviataion (Sd) of 30 measurements, in millimeters, taken from six microscope slide cultures, five measurements/slide culture.

    b - Measurement not made because of experimental design, treatment 2 contained bacteria and mold in constant contact for 24 h.

Table C5. *Penicillium camemberti* hyphal extension in the presence of *B. linens* ATCC 8377 grown upon DMM, DMA, or TYE.

Time (h)	DMA				DMM				TYE			
	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd
0	.11a	.04	.13	.04	.11	.03	.12	.05	.08	.02	.08	.03
12	b		.50	.14	b		.17	.09	b		.08	.02
24	.84	---	.84	---	.14	.05	.15	.07	.08	.02	.09	.03
36	.84	---	.84	---	.16	.06	.14	.06	.10	.02	.09	.02
48					.16	.05	.16	.16	.10	.02	.09	.02
60					.38	.33	.15	.06	.10	.02	.09	.02
72					.42	.36	.15	.05	.09	.02	.10	.03

a
   Mean and standard deviation (Sd) of 30 measurements, in millimeters, taken from six microscope slide cultures, five measurements/slide culture.

   b - Measurement not made because of experimental design, treatment 2 contained bacteria and mold in constant contact for 24 h.

Table C6. *Penicillium camemberti* hyphal extension in the presence of B. linens ATCC 9172 grown upon DMM, DMA, or TYE.

| | Medium | | | | | | | | | | | |
| | DMA | | | | DMM | | | | TYE | | | |
Time (h)	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd
0	.14a	.03	.16	.05	.14	.03	.15	.04	.08	.02	.08	.02
12	b		.83	.08	b		.23	.07	b		.10	.02
24	.84	---	.84	---	.27	.07	.25	.05	.09	.02	.09	.02
36	.84	---	.84	---	.27	.05	.31	.06	.09	.03	.09	.02
48					.28	.05	.31	.06	.24	.12	.10	.02
60					.49	.21	.30	.04	.33	.32	.09	.02
72					.52	.20	.29	.03	.36	.36	.10	.02

a
    Mean and standard deviation (Sd) of 30 measurements, in millimeters, taken from six micro-scope slide cultures, five measurements/slide culture.

    b – Measurement not made because of experimental design, treatment 2 contained bacteria and mold in constant contact for 24 h.

Table C7. Penicillium expansum hyphal extension in the presence of B. linens ATCC 8377 grown upon DMM, DMA, or TYE.

| | Medium | | | | | | | | | | | |
| | DMA | | | | DMM | | | | TYE | | | |
Time (h)	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd
0	.18a	.05	.20	.20	.24	.07	.25	.08	.08	.03	.08	.02
12	b		.83	.08	b		.51	.13	b		.08	.03
24	.84	---	.84	---	.84	---	.84	---	.08	.03	.09	.02
36	.84	---	.84	---	.84	---	.84	---	.10	.03	.09	.02
48									.10	.03	.09	.03
60									.10	.02	.09	.02
72									.09	.02	.10	.03

a
    Mean and standard deviation (Sd) of 30 measurements, in millimeters, taken from six micro-scope slide cultures, five measurements/slide culture.

    b – Measurement not made because of experimental design, treatment 2 contained bacteria and mold in constant contact for 24 h.

Table C8. Penicillium expansum hyphal extension in the presence of B. linens ATCC 9172 grown upon DMM, DMA, or TYE.

| | Medium | | | | | | | | | | | |
| | DMA | | | | DMM | | | | TYE | | | |
Time (h)	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd	Trt 1	Sd	Trt 2	Sd
0	.17a	.05	.17	.05	.14	.05	.15	.06	.18	.10	.31	.16
12	b		.57	.12	b		.24	.06	b		.23	.16
24	.84	---	.84	---	.21	.07	.34	.23	.19	.10	.21	.15
36	.84	---	.84	---	.21	.06	.45	.29	.23	.14	.22	.15
48					.22	.05	.44	.30	.27	.20	.24	.15
60					.84	---	.84	---	.30	.26	.20	.15
72					.84	---	.84	---	.31	.25	.22	.15

a Mean standard deviation (Sd) of 30 measurements, in millimeters, taken from six microscope slide cultures, five measurements/slide culture.

b - Measurement not made because of experimental design, treatment 2 contained bacteria and mold in constant contact for 24 h.

Table C9. Standard error* terms from analysis of
variance of hyphal extension data.

Mold	Strain of B. linens	Standard error
Aspergillus parasiticus	8377	.12
	9172	.15
Aspergillus clavatus	8377	.05
	9172	.13
Penicillium camemberti	8377	.16
	9172	.13
Penicillium expansum	8377	.20
	9172	.09

*
Root mean square error - standard deviation of hyphae
lengths over entire experiment, including all treatments,
media, and times, and not including treatment three or DMA.

Table C9. Standard error* terms from analysis of
variance of hyphal extension data.

Mold	Strain of B. linens	Standard error
Aspergillus parasiticus	8377	.12
	9172	.15
Aspergillus clavatus	8377	.05
	9172	.13
Penicillium camemberti	8377	.16
	9172	.13
Penicillium expansum	8377	.20
	9172	.09

*
Root mean square error - standard deviation of hyphae
lengths over entire experiment, including all treatments,
media, and times, and not including treatment three or DMA.

Table C10. Standard error[*] terms of analysis of
variance of mold spore germination percentages in the presence
of trapping agents and B. linens and in the presence of B.
linens grown upon defined media.

Mold	B. linens strain	Standard error	
		TAa	DMb
Aspergillus parasiticus	8377	8.90	11.30
	9172	7.82	10.69
Aspergillus clavatus	8377	6.86	20.24
	9172	10.96	11.74
Penicillium camemberti	8377	11.38	11.70
	9172	7.46	13.92
Penicillium expansum	8377	8.08	9.89
	9172	14.36	10.15

a
  Root mean square errors from experiments using trapping
agents.

  bRoot mean square errors from experiments using defined
media.

Table C11. Analysis of variance mean squares for hyphal extension data using B. linens ATCC 9172, DMA and treatment three deleted from analysis.

Source of variation	Degrees of freedom	Mean squares[a]			
		Penicillium camemberti	Penicillium expansum	Aspergillus parasiticus	Aspergillus clavatus
Treatment	1	3.64*	605.51	997.24	19.29*
Medium	1	6432.27	17809.17	14723.20	20.49*
Time	5	20.56*	417.45	354.93	558.17
Slide(treat x time)	12	233.81	279.33	164.64	1102.29
Model	20	737.77	1848.78	1688.72	1484.38
Error	b	28.03	29.92	41.46	45.76
Total	b				

[a] Sum of squares/degrees of freedom (df).

* P not significant at .01.

b - Degrees of freedom: 669, P. camemberti; 609, P. expansum; 609, A. parasiticus; 639, A. clavatus. Total degrees of freedom = error df + model df

Table C12. Analysis of variance mean squares for hyphal extension data using B. linens ATCC 8377, DMA and treatment three deleted from analysis.

Source of variation	Degrees of freedom	Mean square[a]			
		Penicillium camemberti	Penicillium expansum	Aspergillus parasiticus	Aspergillus clavatus
Treatment	1	164.37	347.44*	615.40	2.44*
Medium	1	1578.79	12339.88	476.77	48.34
Treatment x medium	1	88.12	329.12*	268.97	109.38
Time	5	9.23*	1605.34	180.85	18.39
Slide(treat x time)	12	87.69	69.90*	906.20	18.73
Model	20	187.33	1675.40	978.61	82.56
Error	b	12.59	70.35	23.96	4.07
Total	b				

[a]
Sum of squares/degrees of freedom (df).

*
P not significant at .01.

b - Degrees of freedom: 639, P. camemberti; 402, P. expansum; 509, A. parasiticus; 424, A. clavatus. Total degrees of freedom = error df + model df

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