Aspects of Enzymology and Biochemical Properties of *Brevibacterium linens* Relevant to Cheese Ripening: A Review[1]

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ABSTRACT

Brevibacterium linens is a major surface microorganism that is present in the smear of surface-ripened cheeses. The enzymology and biochemical characteristics of *B. linens* influence the ripening and final characteristics of smear surface-ripened cheeses. Proteolytic, peptidolytic, esterolytic, and lipolytic activities, which are of particular importance in the ripening process, are discussed in detail. This review also describes the production of volatile compounds, especially sulfur-containing ones, by *B. linens*, which are thought to be important in respect to the flavor of smear surface-ripened cheeses. The unique orange-colored carotenoids and the factors effecting their production by *B. linens* are also presented. The catabolism of aromatic amino acids, bacteriocin production, plasmids, and miscellaneous biochemical and physiological properties (peptidoglycan type, antibiotic resistance, insecticide degradation, and biotechnological applications) of *B. linens* are discussed. The problem associated with the current taxonomical classification of *B. linens* strains caused by strain variation is evaluated. Finally, the application of *B. linens* cell extracts or its proteolytic enzymes as cheese ripening acccelerants for semi-hard or hard cheese varieties is considered.

(**Key words**: *Brevibacterium linens*, cheese, enzymology, biochemical properties)

Abbreviation key: **PHMB** = *p*-hydroxymercuribenzoate, **NA** = nitroanilide.

INTRODUCTION

Brevibacterium linens has long been recognized as an important dairy microorganism because of its ubiquitous presence on the surface of a variety of smear surface-ripened cheeses, such as Limburger, Münster, Brick, Tilsiter, and Appenzeller. The metabolism and physiology of the microorganism determine its growth on smear surface-ripened cheeses and the effect of such growth on the characteristics of the cheese. *Brevibacterium linens* is a strictly aerobic microorganism, with a rod-coccus growth cycle, with temperature growth optimum of 20 to 30°C. *Brevibacterium linens* is a halotolerant microorganism with optimum growth at pH 6.5 to 8.5. The microflora of smear surface-ripened cheeses is generally complex and of a transient nature. Typically, ripening progresses from a yeast and mold flora to a bacterial flora, of which *B. linens* is a major component. Immediately after the manufacture of smear surface-ripened cheese, yeasts dominate the surface microflora; yeasts utilize the lactate present in the curd, leading to an increase in the pH that facilitates the growth of bacteria such as *Arthrobacter* and *Micrococcus* spp., but especially *B. linens* (99). Because of the brine salting of smear surface-ripened cheeses, only halotolerant microorganisms predominate on the surface of the cheese. *Brevibacterium linens* is characterized by a relatively high salt tolerance (15%). The growth of *B. linens* on the surface is thought to be an essential prerequisite for the development of the characteristic color, flavor, and aroma of smear surface-ripened cheeses (1, 28, 56). The growth of *B. linens* is also stimulated by vitamin production by the yeasts during growth (97, 98). The major factors that influence the distinctive characteristics of smear surface-ripened cheeses and the number, type, and growth rate of the surface microflora are the physical and chemical characteristics intrinsic to the cheese (pH, water activity, redox potential, composition, and size), the environmental parameters (ripening temperature, relative humidity) and the technological conditions during manufacture (ripening time, degree of mechanization, and microflora of cheese equipment). This review will cover the taxonomy, biochemistry, and enzymology of *B. linens* with particular emphasis on its importance in relation to bacterial smear surface-ripened cheeses.

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TAXONOMY OF *BREVIBACTERIUM LINENS*

Classification of the genus *Brevibacterium* has presented taxonomists with problems because of its close morphological similarity to other genera, such as *Arthrobacter*, *Caseobacter*, *Corynebacterium*, and *Rhodococcus*. The genus *Brevibacterium* was proposed initially by Breed (14), with *B. linens* as the type species, and was recognized in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (15); no mention was made of coryneform morphology. However, later research showed that *B. linens* had coryneform morphology and showed a rod-coccus cycle similar to that of *Arthrobacter globiformis* (95, 110). In the eighth edition of *Bergey's Manual of Determinative Bacteriology* (106), the genus *Brevibacterium* was listed as *incertae sedis* because of a number of reports indicating its close similarity to the genus *Arthrobacter* and because the coryneform morphology was overlooked by Breed (14); da Silva and Holt (23), Davis and Newton (24), and Bousfield (10) even proposed that *B. linens* should be reclassified as *Arthrobacter linens*.

Later numerical taxonomic (66, 114) and chemotaxonomic (74, 93, 111) studies illustrated the heterogeneity of the group and also indicated that *B. linens* was a distinct taxon that should form the basis of a redefined genus *Brevibacterium*, as was suggested initially by Yamada and Komagata (128) and later by Jones (65), Keddie and Cure (73), and Sharpe et al. (117). On the basis of these and further studies, Collins et al. (21) redefined the genus *Brevibacterium* (Breed), which forms the basis of the genus in the first edition of *Bergey's Manual of Systematic Bacteriology* (67).

At present, the genus *Brevibacterium* contains the type species, *B. linens*, and the species *Brevibacterium iodinum*, *B. casei*, and *B. epidermidis*. However, the situation is further complicated by studies of DNA-DNA homology (40), which indicate heterogeneity exists within the species *B. linens*; DNA-DNA homology studies have shown that those strains presently designated as *B. linens* constitute at least two distinct species. Of the strains studied by Fiedler et al. (40), ATCC 9172 (the type strain), ATCC 19391, ATCC 9864, and strain B3 represent one homology group; this group represents the species *B. linens* because it contains the type strain, ATCC 9172. A second homology group contains strains ATCC 9175, AC 251, AC 252, AC 474, and B4.

The presence of at least two DNA-DNA homology groups within the species *B. linens* is further indicated by considerable differences in the nutritional requirements of different strains (94) and by differences in maximum growth temperature and salt tolerance (117). Another area of difference between the two proposed homology groups is that pigment production is light-dependent for strain ATCC 9172 (type strain and member of DNA-DNA homology group 1) but is light-independent for other strains (including strain ATCC 9175, DNA-DNA homology group 2) (94). Foissy (41), using an electrophoretic zymogram technique, examined intra- and extracellular enzymatic activities in 15 strains of *B. linens*. On the basis of the protein bands, it was possible to divide the 15 strains into three biotypes. By use of a similar electrophoretic zymogram technique, Sørhaug (118) studied intracellular dipeptidase activities in 6 strains of *B. linens*. There were considerable differences in the number of dipeptidases observed for the various strains in addition to differences in substrate specificity, further indicating the heterogeneity between *B. linens* strains. Further heterogeneity within the species was evident in another electrophoretic zymogram study (29) of intracellular esterase activities in 18 strains of *B. linens*; the number of esterase bands varied from 2 to 6, depending on the strain. It is, however, necessary to mention that the expression of these various enzymatic activities may be dependent on the growth medium and environment, and, therefore, any attempted classification of *B. linens* strains based on these reports should be treated with caution.

Characteristics of the Genus *Brevibacterium*

Brevibacterium spp. exhibit a marked rod-coccus cycle during growth on complex media; during the exponential phase, the cells are morphologically rod-shaped, but, as the cells enter the stationary phase of growth, they become coccoid-shaped. Both rod and coccoid forms are Gram-positive, but some strains and older colonies decolorize readily. Some important biochemical and morphological properties of *Brevibacterium* spp. include the following: no endospore formation, nonmotile, optimum growth temperature of 20 to 30 or 37°C (depending on species and strain), obligate aerobes, slight or no acid production from glucose, extracellular proteinase production, catalase-positive, cell-wall peptidoglycan contains meso-diaminopimelic acid (DAP) as the diamino acid, absence of arabinose in the cell wall, absence of mycolic acids, and large amounts of dehydrogenated menaquinone (67).

In the first edition of *Bergey's Manual of Systematic Bacteriology* (67), a number of species are mentioned that almost certainly are not members of the genus

Brevibacterium, but for which data are insufficient to allow them to be reclassified with confidence. Such species are classified as *incertae sedis* and include *Brevibacterium incertum*, *Brevibacterium acetylicum*, *Brevibacterium oxydans*, *Brevibacterium halotolerans*, *Brevibacterium frigoritolerans*, and *Brevibacterium rufescens*.

PROTEOLYTIC AND PEPTIDOLYTIC ACTIVITY

Extracellular, cell-wall associated, and intracellular proteinases have been reported for *B. linens*. For convenience, the proteolytic enzymes of *B. linens* will be discussed according to their location, as extracellular, cell wall-associated, or intracellular.

Extracelluar Proteinase(s)

The greatest number of studies have focused on the extracellular proteinase(s) of the microorganism, largely because of their high activity and importance with respect to cheese ripening. In relation to the extracellular proteinase, some discrepancies exist concerning the number and biochemical properties of the extracellular proteinase(s) of the bacterium. Extracellular proteinase production was observed initially by Albert et al. (2) through the partial hydrolysis of protein in milk cultures. However, the first significant study of extracellular proteinase production by *B. linens* was conducted by Friedman et al. (47), who observed that proteinase production was cyclical and had 2 maxima after 2 and 8 d of growth, in the absence of autolysis, possibly indicating the presence of two distinct enzymes. The proteinase produced after 2 d of growth was studied further and was found to have pH and temperature optima of 7.2 and 38°C, respectively. Neither metals nor reducing agents resulted in loss of activity. The proteinase was active on α-CN and β-CN (crude preparations) with higher activity on the former; no activity was observed on BSA, γ-globulins, β-LG, bovine seminal proteins, or α- LA. Tokita and Hosono (122) reported the partial purification of an extracellular proteinase from *B. linens*, using ammonium sulfate precipitation and gel filtration. The pH and temperature optima of this partially purified proteinase were 7.0 and 25°C, respectively. The proteinase was very heat sensitive and was completely inactivated after exposure to 50°C for 10 min. Casein was hydrolyzed rapidly by the proteinase, hemoglobin was less hydrolyzed, and ovalbumin was hydrolyzed only slightly. In agreement with the results of Friedman et al. (47), cyclical production of the extracellular proteinase was observed.

Brezina et al. (18) reported the partial purification of four extracellular proteinases from *B. linens*. The pH and temperature optima of the proteinases were 5.0 to 8.0 and 50°C, respectively. Inhibition studies indicated that the enzymes were serine proteinases. Juhász and Skárka (69) partially purified an extracellular proteinase from a *B. linens* strain (isolated from the cheese culture, Laktoflora 200; Laktos, Prague, Czech Republic) using a combination of ultrafiltration and gel filtration. The pH and temperature optima of the partially purified enzyme were 7.0 to 8.5 and 45°C, respectively. The proteinase was completely inactivated by heat treatment at 55°C for 30 min. The molecular mass of the partially purified enzyme, as determined by SDS-PAGE, was 52 to 55 kDa. Inhibition studies indicated that the enzyme was a serine proteinase.

Foissy (41) detected extracellular proteinase activity in 15 strains of *B. linens* using an electrophoretic zymogram technique and reported significant differences in the electrophoretic patterns between the strains, indicating heterogeneity within the species. Frings et al. (48) studied the hydrolysis of α_{s1}-CN and β-CN that were present in the growth medium for 5 strains (ATCC 9174, DSM 20158, DSM 20425, DSM 20426, and LBT 102) of *B. linens*. Both SDS-PAGE and HPLC analysis showed qualitative differences in the hydrolysis of the caseins between the stains. For all 5 strains, β-CN was hydrolyzed more rapidly and to a greater extent than was α_{s1}-CN. In a similar experiment using whey proteins in the growth medium, Holtz and Kunz (59) found that 4 strains of *B. linens* (ATCC9174, DSM20158, DSM 20426, and LBT 102) hydrolyzed α-LA more rapidly than did β-LG. Hayashi et al. (51) purified five extracellular proteinases from *B. linens* F to homogeneity, using ammonium sulfate precipitation, gel filtration, and anion-exchange chromatography. The isolated proteinases, designated A, B, C, D, and E, had molecular masses of 37, 37, 44, 127, and 325 kDa, respectively, as determined by gel filtration. The enzymes were classified into two groups based on their temperature optima and stability. Proteinases A and B were stable for 1 h at 35°C and had a temperature optimum at 40°C, and proteinases C, D, and E were stable for 1 h at 45°C and had a temperature optimum at 55°C. The pH optimum for all 5 proteinases was 11.0, and proteinases A and B were more active at lower pH values than were proteinases C, D, and E. The 5 proteinases were stable between pH 6.0 to 11.0. Inhibition studies indicated that the isolated enzymes were serine proteinases. Rattray et al. (100) purified an extracellular proteinase for *B. linens* ATCC 9174 with pH and temperature optima

TABLE 1. Biochemical properties of extracellular proteinase(s) of *Brevibacterium linens*.

Strain	No.	Temperature optimum	pH optimum	Molecular mass (kDa)	Type	Reference
Bacterium linens 450	2	38	7.2	ND[1]	ND	(47)
B. linens (unspecified)	1	25	7	ND	ND	(122)
B. linens Lactoflora 200	1	45	7.0–8.5	52–55[2]	Serine	(69)
B. linens Laktoflora 200	4	50	5.0–8.0	ND	Serine	(18)
B. linens F	5	40–55	11	37–325[3]	Serine	(51)
B. linens ATCC 9174	1	50	8.5	56[2], 126[3]	Serine	(100)

[1]Not determined.

[2]Determined by SDS-PAGE.

[3]Determined by gel filtration.

of 8.5 and 50°C, respectively. The molecular mass of the proteinase was 56 kDa by SDS-PAGE and was 126 kDa by gel filtration, indicating that the native enzyme exists as a dimer. Inhibition studies indicated that the enzyme was a serine proteinase. The enzyme was activated by Mg^{2+} and Ca^{2+}. The sequence of the first 20 N-terminal amino acids was reported. The specificity of the proteinase on bovine α_{s1}-CN and β-CN was characterized (103, 104). The time course of peptide formation from α_{s1}-CN indicated that His_8-Gln_9, Ser_{161}-Gly_{162}, and Gln_{172}-Tyr_{173} or Phe_{23}-Phe_{24} were the first, second, and third bonds cleaved, respectively. Other cleavage sites in α_{s1}-CN included Asn_{19}-Leu_{20}, Phe_{32}-Gly_{33}, Tyr_{104}-Lys_{105}, Leu_{142}-Ala_{143}, Phe_{150}-Arg_{151}, Gln_{152}-Phe_{153}, Leu_{169}-Gly_{170}, and Thr_{171}-Gln_{172}. The major sites of hydrolysis of β-CN were Ser_{18}-Ser_{19}, Glu_{20}-Glu_{21}, Gln_{56}-Ser_{57}, Gln_{72}-Asn_{73}, Leu_{77}-Thr_{78}, Ala_{101}-Met_{102}, Phe_{119}-Thr_{120}, Leu_{139}-Leu_{140}, Ser_{142}-Trp_{143}, His_{145}-Gln_{146}, Gln_{167}-Ser_{168}, Gln_{175}-Lys_{176}, Tyr_{180}-Pro_{181}, and Phe_{190}-Leu_{191}. The proteinase showed broad specificity on both α_{s1}-CN and β-CN for the amino acids present in the P_1 and P'_1 positions but showed a general preference for hydrophobic residues at the P_2, P_3, P_4, P'_2, P'_3, and P'_4 positions.

These various reports show that there is poor agreement as to the characteristics or the number of the extracellular proteinase(s) of *B. linens*. Strain variation or autoproteolysis may provide a possible explanation. The biochemical properties of the extracellular proteinase(s) of *B. linens* are summarized in Table 1.

Induction of Extracellular Proteinase Production

A number of attempts have been made to stimulate the production of extracellular proteinases by *B. linens* by modification of the growth conditions. Friedman et al. (47) found that the inclusion of glucose in the growth medium resulted in no appreciable increase in extracellular production of the proteinase. Tokita and Hosono (122) observed a similar effect. Zemanovic and Shárka (129) found that egg albumen and zein (corn gluten) in the culture medium were the most effective in stimulating proteinase production; casein was the poorest stimulant. The effect of temperature on proteinase production has also been studied. Tokita and Hosono (122) and Zemanovic and Skárka (129) observed that increasing the cultivation temperature from 25 to 30°C caused a 70% decrease in the production of extracellular proteinase production by *B. linens*. However, Hayashi et al. (52) found no significant change in proteinase production at elevated growth temperatures. Production of extracellular proteinase also appears to be influenced by the pH of the growth medium prior to inoculation. Friedman et al. (47) reported that an initial pH of 7.0 resulted in maximum proteinase production, and Zemanovic and Skárka (129) reported that an initial pH of 8.0 to 8.5 was optimal; in both studies, proteinase production was lowest when the initial pH of the medium was 6.0.

Extracellular Aminopeptidases

Purification, to homogeneity, of an extracellular aminopeptidase from the cell-free supernatant of *B. linens* ATCC 9174 was reported by Foissy (42, 43, 44, 45); no activity of the intracellular marker enzyme, glucose-6-phosphate dehydrogenase, was detected in the cell-free supernatant, indicating that cell lysis had not occurred during growth and that the purified aminopeptidase was truly extracellular. The purification protocol involved ammonium sulfate precipitation, followed by gel filtration, reprecipitation with ammonium sulfate, and finally, two preparative electrophoresis steps. The purified aminopeptidase had

pH and temperature optima of 9.6 and 28°C, respectively. The molecular mass of the purified extracellular aminopeptidase, determined by gel filtration and SDS-PAGE, was 95 and 48 kDa, respectively, indicating that the aminopeptidase exists as a dimer in its native state. Activation and inhibition studies showed that Co^{2+} resulted in pronounced activation of the aminopeptidase. Metal-chelating agents, reducing agents, and *N*-bromosuccinimide inhibited the aminopeptidase. Specificity studies showed that the extracellular aminopeptidase had a strong preference for leucine at the N-terminal of dipeptides with much less activity when phenylalanine, serine, or histidine was at the N-terminal position. The aminopeptidase did not hydrolyze dipeptides with lysine, glycine, or proline at the N-terminus.

Hayashi and Law (53) reported the purification of two extracellular aminopeptidases from *B. linens* F, which were designated aminopeptidase A and B. Aminopeptidase A accounted for 85% of the aminopeptidase activity remaining at the end of the purification procedure. Both aminopeptidases had a pH optimum of 9.3 and a temperature optimum of 40°C; aminopeptidase A showed slightly greater pH stability than did aminopeptidase B. The molecular masses of aminopeptidases A and B were estimated as 150 and 110 kDa, respectively, by gel filtration, and as 36 and 26 kDa, respectively, by SDS-PAGE, indicating that the aminopeptidases exist as tetramers in their native state. Aminopeptidases A and B were completely inhibited by EDTA and were reactivated on incubation with Ca^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}, or Mn^{2+}, indicating that the aminopeptidases are metalloenzymes. The two aminopeptidases had similar substrate specificities with a strong preference for Leu-*p*-nitroanilide (**NA**) and dipeptides with leucine at the N-terminus. In addition to activity on dipeptides, the aminopeptidases also hydrolyzed tri- and tetrapeptides and dipeptides with proline at the N-terminus.

Brezina et al. (18) reported the presence of three extracellular aminopeptidases from a *B. linens* strain isolated from the cheese culture Laktoflora 200. The partially purified aminopeptidases had pH and temperature optima at 7.0 to 9.0 and 30°C, respectively. The enzymes were inhibited by EDTA, Zn^{2+}, Cu^{2+}, or Ni^{2+}; Co^{2+} increased activity by 100%.

Intracellular and Cell-Wall–Associated Proteolytic Enzymes

The presence of intracellular proteinase activity in *B. linens* has also been reported (41, 47, 48). The intracellular proteinase activity reported was measured using casein as substrate; however, the intracellular proteinase activity was relatively low compared with the extracellular proteinase activity. Foissy (41) reported that several strains produced up to four distinct intracellular proteinases, but some strains produced only two. Wong and Cone (127) also demonstrated proteolytic activity in a crude cell-free extract of *B. linens*; enzyme activity was maximal at pH 7.9 and at 45°C. The crude cell-free extract was affected little by reducing agents, iodoacetic acid, *N*-ethylmaleimide, or EDTA, but activity was markedly reduced by Hg^{2+} and *p*-hydroxymercuribenzoate (**PHMB**).

El-Soda et al. (30) partially purified an intracellular aminopeptidase from *B. linens* HS using gel filtration. The pH and temperature optima of the partially purified intracellular aminopeptidase were 7.5 and 30°C, respectively. The enzyme was inhibited with increasing effectiveness by 1,10-phenanthroline > PHMB > phenylmethylsulfonyl fluoride. The enzyme was active on Gly- and Ala-*p*-NA with greater activity on the former, but the enzyme did not hydrolyze Leu-, Lys-, Pro-, or Arg-*p*-NA. The crude cell-free extract showed no dipeptidylaminopeptidase activity on Arg-Pro-, Gly-Pro-, or Gly-Phe-*p*-NA. Sørhaug (118), using an electrophoretic zymogram technique, demonstrated intracellular dipeptidase activity in 6 strains of *B. linens*. A total of 18 different dipeptidase bands was observed for the 6 strains. The greatest number of dipeptidases, 14, was observed for *B. linens* ATCC 21330 and the least, 7, for *B. linens* ATCC 9172. There were considerable differences in substrate specificity between strains. Rattray and Fox (101) purified an intracellular aminopeptidase from *B. linens* ATCC 9174. The pH and temperature optima for this enzyme were 8.5 and 35°C, respectively. The molecular mass was reported to be 59 kDa by SDS-PAGE and 69 kDa by gel filtration. The aminopeptidase was strongly inhibited by PHMB, Co^{2+}, and Zn^{2+} and hydrolyzed Ala-*p*-NA and Gly-*p*-NA but not by Val-, Phe-, Pro-, Glu-, Leu-, Lys-, Arg-, Gly-Phe-, or Ala-Pro-*p*-NA. The enzyme also hydrolyzed dipeptides with an alanine residue in the N-terminal position, but tripeptides were not hydrolyzed. The N-terminal amino acid sequence of the first 20 amino acid residues of the enzyme was also reported.

Ezzat et al. (33) reported cell-wall–associated proteinase and dipeptidase activities in *B. linens* CNRZ 944. Extraction of cell-wall–associated enzymatic activities involved washing the cell pellet initially with a calcium-containing buffer at 4°C and subsequent washing with a calcium-free buffer at 30°C to release the enzymes. The cell wall-associated proteinase was partially purified; preliminary characterization

demonstrated pH and temperature optima of 6.5 and 40°C, respectively. The cell-wall–associated proteinase was inhibited by EDTA, PHMB, and phenylmethylsulfonyl fluoride.

LIPOLYTIC AND ESTEROLYTIC ACTIVITIES OF *BREVIBACTERIUM LINENS*

Foissy (41) demonstrated extracellular esterase activity in 14 strains of *B. linens*, but activity was low compared with intracellular activity. However, Sørhaug and Ordal (119) detected no extracellular esterase activity in 5 strains of *B. linens*, perhaps because of low assay sensitivity. *Brevibacterium linens* I has been reported to have extracellular lipase activity (108), but, again, no such activity was found by Sørhaug and Ordal (119). Cell-wall–associated esterase activity in *B. linens* has also been reported (33, 119). Brandl and Petutschnig (13) found a relationship between the proteolytic and lipolytic activity for *B. linens*; strains with medium or strong proteolytic activity also had relatively high lipolytic activity. Intracellular esterase activity in 15 strains of *B. linens* was observed by Foissy (41) using α-naphthyl acetate, β-naphthyl butyrate, and tributyrin as substrates. Several esterase bands were noted for all of the strains when α-naphthyl acetate or β-naphthyl butyrate was used as substrate, but a single esterase band was found when tributyrin was used (except for 1 strain that had no activity on tributyrin). El-Shafei et al. (29) reported intracellular esterolytic activity in 18 strains of *B. linens*. All strains tested hydrolyzed *o*-nitrophenyl and *p*-nitrophenyl derivatives of acetic and butyric acids, but the derivatives of caprylic and palmitic acids were not hydrolyzed. It was also noted that the *p*-nitrophenyl esters of the fatty acids were hydrolyzed faster than were the *o*-nitrophenyl derivatives. Using an electrophoretic zymogram technique, it was determined that the number of esterase bands varied from 2 to 6, depending on the strain, and most stains showed 4 active esterases.

Lambrechts et al. (82), using an electrophoretic technique, identified 7 esterase bands in the cell-free extract of *Brevibacterium* sp. R312. Eight esterases were separated by anion-exchange chromatography, and the three principal esterases (designated as esterase 4b, 2, and 4a) were purified to homogeneity using anion-exchange chromatography, gel filtration, and affinity chromatography. The molecular masses of esterases 4b, 2, and 4a were 38, 45, and 56 kDa, respectively, as determined by SDS-PAGE. The three esterases differed in their temperature optima and thermal stability; all were active in the same pH range (5.5 to 8.6). Esterase 2 differed from the two other esterases in its sensitivity to inhibitors. Esterase 4b differed from esterases 2 and 4a in its substrate specificity; it hydrolyzed aliphatic and nitrophenyl esters. The spectrum of activity of the other two esterases was narrower, and they hydrolyzed only naphthyl esters and, in the case of esterase 2, tributyrate and ethyl butyrate. Rattray and Fox (102) purified and characterized an intracellular esterase from *B. linens* ATCC 9174. The pH and temperature optima were 7.5 and 35°C, respectively. The molecular mass was found to be 54 kDa by SDS-PAGE and 201 kDa by gel filtration. The esterase hydrolyzed β-naphthyl esters of acetic, butyric, caproic, caprylic, and capric acids but not lauric, myristic, palmitic, or oleic acids. The sequence of the first 19 N-terminal amino acids of the esterase was determined.

PRODUCTION OF VOLATILE COMPOUNDS

Sulfur-Containing Compounds

Tokita and Hosono (120) studied the production of volatile sulfur compounds by *B. linens* in the culture medium. The volatile sulfur compounds were identified as hydrogen sulfide, mercaptans, and disulfides; hydrogen sulfide was at the highest concentration. Methanethiol was also detected. The addition of methionine to the culture medium dramatically increased the level of hydrogen sulfide produced, but the addition of cystine and cysteine had little effect. Sharpe et al. (116) detected the production of methanethiol by 7 strains of *B. linens* and suggested that because *B. linens* is a major component of smear surface-ripened cheeses, such as Limburger, Roquefort, and Stilton, these methanethiol-producing bacteria may contribute to the aroma and flavor of these cheeses.

Cuer et al. (22) studied the production of sulfur-containing compounds by 8 strains of *B. linens*. All 8 strains produced large amounts of methanethiol, hydrogen sulfide, dimethyldisulfide, and 2,3,4-trithiapentane. Enrichment of the culture medium with methionine resulted in increased production of methanethiol, and the addition of cysteine increased the production of hydrogen sulfide. Four of the strains also produced *S*-methylthioacetate, which is an important contributor to the characteristic odor of smear surface-ripened cheeses. Lamberet et al. (80, 81) examined in some detail the ability of *B. linens* to synthesize various *S*-methyl thioesters by incubating resting cells with methanethiol in the presence and absence of short-chain fatty acids; *S*-methyl thioacetate, *S*-

methyl thiopropionate, *S*-methyl thioisobutyrate, *S*-methyl thio-2-methylbutyrate, and *S*-methyl thioisovalerate were produced by the organism when incubated with methanethiol alone. However, when short-chain fatty acids were included together with the methanethiol, *S*-methyl thiobutyrate, *S*-methyl thiovalerate, and *S*-methyl thiocaproate were also produced. The production of such *S*-methyl thioesters by *B. linens* could be through the activity of several intracellular acyltransferases or through the reverse action of esterases. Lamberet et al. (81) also showed that *B. linens* produced a greater quantity and larger number of the different *S*-methyl thioesters compared with lactic acid bacteria; considerable strain variation was also demonstrated for the *B. linens* strains tested.

Hemme and Richard (57) isolated 80 bacterial strains from the surface of Camembert cheeses and tested them for their capacity to produce methanethiol. The only strains able to produce significant quantities of methanethiol were orange coryneform bacteria, which may be related to *B. linens*. The methanethiol-producing strains could grow in a minimal medium containing L-methionine or α-ketobutyrate.

Hemme et al. (56) showed that, when *B. linens* CNRZ 918 was cultivated in a complex medium containing relatively high levels of methionine (0.1%), methanethiol production reached maximum levels in the late exponential phase of growth, at which point the cells are rod-shaped. A detailed series of studies on the production of methanethiol by *B. linens* CNRZ 918 was conducted by Ferchichi et al. (37, 38, 39). In confirmation of the work of Hemme et al. (56), it was found that maximum production of methanethiol occurred during the exponential phase of growth, when the cells were rod-shaped (39). The production of methanethiol by *B. linens* was significantly enhanced by the presence of methionine in the medium, probably reflecting the induction of the enzymatic systems involved. It was also found that glucose favored growth but inhibited the production of methanethiol, and lactate favored both growth and methanethiol production. It was also shown that methanethiol production was maximal under growth conditions that were not optimal (37). The induction of methanethiol production showed that the lowest level of L-methionine capable of inducing maximum methanethiol production was 7 m*M* (38). The peptides L-Ala-L-Met and L-Met-L-Ala induced greater methanethiol production than did free L-methionine, probably because of better cell penetration by the dipeptides. D-Methionine, L-cysteine, *S*-methyl-L-cysteine, and L-ethionine were poor inducers. A Na^+ concentration of 1 *M* in the culture medium led to maximum methanethiol production. The induction of methanethiol production is probably due to the induction of L-methionine-γ-demethiolase, and the transport system for L-methionine by *B. linens* was found to be constitutive and dependent on Na^+ (38). The demethiolation activity of a crude cell extract of *B. linens* showed that the activity was thermolabile and was strongly inhibited by Zn^{2+}, Mn^{2+}, and Cu^{2+}. L-Methionine and L-ethionine were the best substrates for the crude cell extract (with the production of methanethiol and ethanethiol, respectively). Methanethiol was not produced by the crude cell-free extract from D-methionine, L-methioninamide, L-Ala-L-Met, or L-Met-L-Ala (38).

Collin and Law (20) partially purified and characterized the L-methionine-γ-demethiolase from *B. linens* NCDO 739. The temperature and pH optima for the enzyme were 20°C and 8.0, respectively. The partially purified enzyme was stable between pH 5.5 and 7.5. The native molecular mass (by gel filtration) and the pI of the L-methionine-γ-demethiolase were 175 kDa and 5.0 to 6.0, respectively. Recently, the purification of this enzyme to homogeneity has been reported (27); the enzyme catalyses the α,γ elimination of methionine to produce methanethiol, α-ketobutyrate, and ammonia and requires pyridoxal phosphate as a cofactor. The native molecular mass was 170 kDa, consisting of 4 identical subunits of 43 kDa. The enzyme was active at the salt concentration and pH found in Cheddar cheese but was susceptible to degradation by intracellular proteases. The enzyme was induced by the addition of methionine to the growth medium (26). Cystathionine β-lyase activity in whole cells and cell-free extracts of *B. linens* has also been reported, but the activity was low in comparison with the L-methionine-γ-demethiolase activity (25).

Weimer et al. (126) measured the methanethiol-producing capacity of *B. linens* and lactic acid bacteria in optimum (pH 8.0, 0% NaCl) and cheese-like conditions (pH 5.2, 4.0% NaCl). The methanethiol-producing capacity of *B. linens* was significantly higher than that observed for the lactic acid bacteria under both conditions. It was also found that the addition of Met-containing peptides or peptides formed from the hydrolysis of β-CN to the medium during growth of *B. linens* induced the methanethiol-producing capacity of the cells.

An interesting phenomenon regarding the production of methanethiol by *B. linens*, in addition to flavor and aroma implications for smear surface-ripened cheeses, is the toxicity of methanethiol toward molds. Beattie and Torrey (8) reported that methanethiol

produced by *B. linens* ATCC 8377 inhibited the germination of spores of *Penicillium expansum* NRRL 877, which possibly explains why there is a notable lack of ability of Limburger and Trappist cheeses to support mold growth. Lewis (88) investigated the antimicrobial activity of 4 strains of *B. linens* against *Pencillium roqueforti*. Only those three strains that produced methanethiol were shown to be inhibitory against the mold indicating the toxicity of methanethiol toward molds.

Other Volatile Compounds

Tokita et al. (123), in a preliminary study on the production of volatile compounds by *B. linens*, identified the presence of volatile acids, volatile bases, and neutral substances in the culture medium. From gas chromatography data, acetic, isovaleric, and caproic acids were found to be the principal volatile compounds. Volatile amines were also identified, including histamine, tyramine, dibutylamine, monoethylamine, monomethylamine, diethylamine, and cadaverine. The neutral volatile substances included formaldehyde, acetaldehyde (acetoaldehyde), acetone (aceton), ethanol, isopropanol, *n*-propanol, and isobutanol.

Hosono and Tokita (61, 63) studied the production of volatile flavor compounds by *B. linens*, *Candida mycoderma*, and *Debaryomyces kloekeri* in broth medium. When grown in milk broth, *B. linens* produced higher levels of hydrogen sulfide, volatile fatty acids (particularly *n*-butyric acid), and volatile carbonyl compounds (formaldehyde, acetaldehyde, acetone, pentanone-2, and heptanone-2) than did *C. mycoderma* or *D. kloeckeri*. It was also shown that *B. linens* produces acetone, acetaldehyde, and acetic acid from citric acid. Hosono and Tokita (62) reported that larger amounts and a greater number of volatile carbonyl compounds were produced by *B. linens* from casein than from milk fat or glucose, indicating that casein is an important source for the production of volatile carbonyl compounds.

In another study, Hosono and Tokita (60) studied the decarboxylation of 13 amino acids and subsequent amine production by a crude cell extract from *B. linens*. The amines produced from lysine, alanine, leucine, glutamate, and tyrosine were cadaverine, monomethyl amine, isoamyl amine, γ-amino butyric acid, and tyramine, respectively; lysine was decarboxylated at the highest rate. Hemme et al. (56) studied the deamination of amino acids by 23 strains of *B. linens* isolated from the smear of Comté and Beaufort cheeses. Serine, glutamine, and asparagine were actively deaminated by certain strains. Most strains exhibited very low deaminating activity on the other amino acids. The deamination of phenylalanine, tryptophan, and histidine could not be demonstrated.

Jollivet et al. (64) conducted a comprehensive study on the production of volatile compounds by 4 strains of *B. linens*. The 4 strains produced a wide range of compounds belonging to different chemical families: fatty acids, alcohols, methyl ketones, pyrazines, sulfur compounds, and cyclic compounds. Both quantitative and qualitative differences existed between the compounds produced by the different strains. The strains produced 2,5-dimethylpyrazine in sufficiently high concentrations for flavor perception; this compound is present in several cheeses, such as Camembert, and gives a nutty, roasted note. The important flavor compound, dimethyltrisulfide, was produced by all 4 strains at concentrations higher than its sensory threshold; its odor is that of very ripe cheese.

CATABOLISM OF AROMATIC AMINO ACIDS

Brevibacterium linens may participate in the formation of flavor compounds and their precursors through the catabolism of aromatic amino acids. Such compounds include phenol and indole, both of which are found at high concentrations in Limburger cheese (96).

The transport of radioactively labeled phenylalanine, tyrosine, and tryptophan by *B. linens* has been studied (11). Based on the differential behavior of the transport in terms of concentration-dependent kinetics, pH and temperature optima, structural and stereospecificity, and responses to metabolic inhibitors and sulfydryl reagents, it was concluded that the transport of aromatic amino acids by *B. linens* is determined by three high affinity permeases. The transport of phenylalanine was optimal at pH 7.5 and 25°C and that of tyrosine and tryptophan at pH 8.0 and 35°C. Tryptophan noncompetitively inhibited the transport of tyrosine; similarly, phenylalanine and tyrosine noncompetitively inhibited the transport of tryptophan. All combinations of the aromatic amino acids resulted in noncompetitive inhibition of the transport mechanism. Transport was almost totally inhibited by carbonyl cyanide-*m*-chlorophenylhydrazone and 2,4-dinitrophenol, indicating that the prime energy source for transport was proton conduction.

The transport of phenyalanine by *B. linens* was studied further by Boyaval et al. (12) using high resolution autoradiography. It was shown that labeled phenylalanine rapidly penetrates the cells and

is localized in the cytoplasm; the labeled phenylalanine did not remain bound to the cell wall or membrane. It was not possible to identify the precise localization of the transported phenylalanine in the cytoplasm because of the small size of the cells. It was also noted that chromatography of bacterial extracts gave two radioactive spots, one that was due to phenylalanine and the other due to a small peptide.

Lee and Desmazeaud (84) reported that transamination was the first step in the utilization of aromatic amino acids as sole nitrogen sources by *B. linens*. The deaminated metabolites of the amino acids were identified in culture supernatants, and the enzyme activity was identified in cell-free extracts. They also reported that the cells contained increased aromatic amino acid aminotransferase activity when aromatic amino acids were used as sole nitrogen sources. In addition, two aromatic amino acid aminotransferases from a cell-free extract were resolved by ion exchange chromatography. One of these aromatic amino acid aminotransferases was purified and had pH and temperature optima of 8.5 to 9.0 and 37 to 40°C, respectively (85). The molecular mass of the enzyme was estimated by gel filtration to be 126 kDa. The cofactor pyridoxal 5-phosphate was tightly bound to the enzyme.

Lee et al. (87) investigated the enzymatic mechanisms involved in the catabolism of phenylalanine and tyrosine by some coryneform bacteria isolated from cheese; the two key steps studied were deamination and benzene ring cleavage. It was found that orange coryneforms catabolized phenylalanine and tyrosine by transamination, and the benzene ring was cleaved by 3,4-dihydroxyphenylacetate-2,3-dioxygenase; both enzymes appeared to be inducible. It was also noted that yellow and white coryneforms (in contrast to orange-pigmented coryneforms) possessed uninducible low aminotransferase activity and lacked enzymes for benzene ring cleavage. In a further study (86), the quantitative importance of the 3,4-dihydroxyphenylacetate *meta*-cleavage pathway (the principal pathway of tyrosine catabolism) in the catabolism of phenylalanine in *B. linens* 47 was evaluated using tyrosine-negative mutants. Less than 5% of phenylalanine was catabolized through the tyrosine pathway, indicating that in *B. linens* the two structurally analogous amino acids are catabolized principally by different pathways.

PRODUCTION OF BACTERIOCINS AND ANTIMICROBIAL SUBSTANCES

Grecz et al. (50) demonstrated the ability of culture supernatants from *B. linens* (ATCC 9174 and ATCC 9175) to inhibit the germination of spores of *Clostridium botulinum* type A. The inhibitory agent present in the culture fluid was not identified, but the maximum inhibitor level was produced after 7 d of growth. The unidentified inhibitory substance was active after heat treatment at 121°C for 15 min; it was soluble in 70% ethanol and was always associated with a yellow-red color. Culture supernatants from strain ATCC 9175 were more active than were those from strain ATCC 9174. The production of this inhibitory substance may explain why surface-ripened cheeses are free of *C. botulinum*.

Bacteriocin production by *B. linens* was demonstrated by Kato et al. (72). The bacteriocin, designated as Linecin, was produced by *B. linens* ATCC 9175 and another *B. linens* strain (unspecified) and was found to be inhibitory to *B. linens* ATCC 8377 and *B. linens* ATCC 9172. Linecin was not inhibitory to other species within the genus *Brevibacterium*, *Corynebacterium*, or *Micrococcus*. In a later investigation, Kato et al. (70) reported the purification and characterization of Linecin A from the culture supernatant of *B. linens* ATCC 9175. Linecin A consisted mostly of protein with an estimated molecular mass of 95 kDa (as determined by gel filtration). Linecin A was also sensitive to proteolytic attack, thermolabile, and totally inactivated by heating at 45°C for 60 min. Linecin A inhibited *B. linens* strains ATCC 8377, ATCC 9172, and ATCC 9174.

Valdés-Stauber and Scherer (124) isolated and characterized a bacteriocin, designated Linocin M18, from the culture supernatant of *B. linens* M18 (isolated from the surface of red smear cheese). The bacteriocin consisted of a single protein subunit with a molecular mass of 31 kDa by SDS-PAGE, but in its native state formed aggregates of extremely high molecular masses (>2000 kDa). Linocin M18 was heat labile (totally inactivated after 5 min at 80°C) and was sensitive to a wide range of proteinases. The N-terminal amino acid sequence was determined. Usually, bacteriocins inhibit only closely related bacteria, but Linocin M18 exhibited an extraordinarily broad activity spectrum with activity against species of the genera *Bacillus, Arthrobacter, Corynebacterium, Micrococcus,* and *Listeria*. Of particular interest is the inhibition of *Listeria* spp., which may be important in the biological control of pathogenic *Listeria* spp. in smear surface-ripened cheeses. Oligonucleotide probes based on the N-terminal amino acid sequence have been used to locate the gene coding for Linocin M18 (125). A single copy of the gene, *lin*, was located on chromosomal DNA. The amino acid composition, N-terminal sequence, and

TABLE 2. Properties of bacteriocins and antimicrobial agents of *Brevibacterium linens*.

Name	Inhibitory against	Thermal stability	Molecular mass (native kDa)	Reference
Inhibitory agent	*Clostridium botulinum*	Active after 15 min at 121°C	ND[1]	(50)
Linecin A	*B. linens*	Inactive after 60 min at 45°C	95	(70, 72)
Linocin M18	*Bacillus, Arthrobacter, Corynebacterium, Micrococcus, Listeria*	Inactive after 5 min at 80°C	>2000	(124)
Antimicrobial agent	*Listeria*	Active after 30 min at 80°C	<1	(92)
Linenscin OC2	*Staphylococcus aureus, Listeria monocytogenes*	50% activity lost after 10 min at 100°C	>285	(90)

[1]Not determined.

molecular mass derived from the nucleotide sequence of an open reading frame of 798 nucleotides coding for 266 amino acids found on a 3-kb *Bam*HI restriction fragment correspond closely to those obtained from the purified bacteriocin. The taxonomic distribution of *lin* was also studied; 52 isolates of different species of the genera *Brevibacterium*, *Arthrobacter*, and *Corynebacterium* were probed for *lin* by polymerizing chain reaction. The gene could be amplified in a surprisingly wide distribution of bacteria; it was amplified in 12 of 26 *B. linens* strains isolated from different cheeses, in 1 strain of *Brevibacterium flavum*, 1 strain of *Brevibacterium lyticum*, 5 of 6 *Arthrobacter* spp., and 5 of 9 *Corynebacterium* spp. In a model cheese ripening system, the antimicrobial activity of *B. linens* M18 toward *Listeria* strains has been demonstrated with reduction of *Listeria ivanovii* and *Listeria monocytogenes* counts by 1 to 2 log units (31).

In another study, 3 isolates of *B. linens* (isolated from the brine used to salt red smear cheese) have been shown to produce an antimicrobial agent active against *Listeria* spp. (92). The antimicrobial agent was dialyzable through a membrane with a 1-kDa cutoff, was stable at pH 4.0 to 9.0, and was stable to heat treatment for 30 min at 80°C at acid pH. The agent also remained active after treatment with proteinase, catalase, or lipase. The antimicrobial agent was bacteriostatic or bacteriocidal, depending on the strain of *L. monocytogenes* tested. In a similar study, Ryser et al. (107) isolated and identified from cheese an orange coryneform resembling *B. linens* that produced a bacteriocin-like agent that was inhibitory for *Listeria* spp. including *L. monocytogenes*. The bacteriocin-like agent was resistant to 5 different proteolytic enzymes.

Maisnier-Patin and Richard (90) purified to homogeneity an antibacterial substance, designated Linenscin OC2, from the cell-free supernatant of *B. linens* OC2. This antibacterial substance was different from Linencin A and Linocin M18 and is not a bacteriocin in the strict sense. The antibacterial substance was inhibitory toward foodborne pathogens, including *Staphylococcus aureus* and *L. monocytogenes*, but not against Gram-negative bacteria. Hemolytic activity of the substance on sheep erythrocytes was also demonstrated by Boucabeille et al. (9). The molecular mass of the native Linenscin OC2 was estimated with gel filtration to be 285 kDa; however, SDS-PAGE and mass spectrometry indicated molecular masses of 1196.7 Da and 2412 Da, respectively, indicating that the native Linenscin OC2 exists as large aggregates (90). About 50% of the activity remained after heating for 10 min at 100°C. The antibacterial activity of crude Linenscin OC2 was reduced but not eliminated by incubation with various proteolytic enzymes, α-amylase, and lipase (90). The biochemical mode of action of Linenscin OC2 is believed to be similar to that of bacteriocins such as nisin, which in addition to their cytoplasmic membrance-disruptive action induce autolysis (9). The biochemical properties of the various bacteriocins and antibacterial agents produced by *B. linens* are summarized in Table 2.

One aspect of particular importance with respect to the production of these various bacteriocins and antibacterial substances by *B. linens* is the extrapolation of the inhibitory effects from a model buffer system to that of a smear surface-ripened cheese. The degree to which a bacteriocin produced at the surface of the cheese by *B. linens* can diffuse toward the center obviously influences any inhibitory effect on microorganisms in the interior of the cheese. The molecular masses of the bacteriocins reported from *B. linens* are probably too large to result in significant diffusion from the surface to the interior, and their effects are, therefore, likely to be confined to the surface only.

PLASMIDS

Only a few studies have focused on the genetics of *B. linens*, mainly because most dairy microbiologists have tended to concentrate on the genetics of the commercially more important bacteria such as the lactic acid bacteria. Nevertheless, Sandoval et al. (109) screened a wide selection of species of the genus *Brevibacterium* for the presence of plasmid DNA. A plasmid was isolated from *B. linens* CECT75, designated as pBL100, which is a multicopy plasmid with a size of 7.75 kb. The restriction map of pBL100 showed that it had single restriction sites for the endonucleases *Hind*III, *Pst*I, *Bgl*II, *Eco*RI, and *Bam*HI. The function of this plasmid is unknown, but the authors speculated that it did not appear to code for bacteriocins or antibiotic resistance. In a further study of the genus *Brevibacterium*, Kato et al. (71) purified plasmid pBL 33 from *B. linens* ATCC 9174, which had a size of 7.5 kb. The function of pBL 33 was not determined. The restriction map of plasmid pBL 33 was very similar to that of pBL100 isolated from *B. linens* CECT75 (109).

Holtz et al. (58) screened six strains of *B. linens* for the presence of plasmids. Plasmids of 7.5 (pBL 100) and 7.8 (pBL 33) kb were purified from strains DSM 20158 and ATCC 9174, respectively. These plasmids were the same as those purified by Sandoval et al. (109) and by Kato et al. (71), for which the phenotypic markers were not identified. Comparison of the restriction maps of pBL 100 and pBL 33 showed that the two plasmids were similar, except for a difference in size of 0.3 kb. The deletion zone was found between the restriction sites for the endonucleases *Bgl*II and *Hind*III. No plasmids were found in the other four strains. Phenotypic studies tentatively indicated that resistance to the antibiotic, kanamycin, is probably conferred by pBL 100 but not by pBL 33. It was tentatively suggested that pBL 100 codes for the extracellular proteinase of *B. linens*.

PIGMENTATION

One of the main characteristics in addition to flavor for consumer acceptability of smear surface-ripened cheese is the color of the rind. For cheese varieties such as Saint Paulin, St. Nectaire, Limburger, Tilsiter, and Münster the characteristic red-orange color of the rind is particularly necessary. This characteristic color of the rind is correlated with the shift from the dominant yeast and mold microflora in the early stages of ripening to the bacterial microflora in the later stages of ripening (28, 91). The red-orange color of the smear is primarily due to the pigments produced by *Brevibacterium* sp., *Corynebacterium* sp., *Micrococcus* sp., and *Arthrobacter* sp. (28, 113). The precise contribution that any one species has on determining the final color of a smear surface-ripened cheese is difficult to determine due to the complexity of the surface flora, but *B. linens* is recognized as one of the main contributors.

Albert et al. (2) investigated the effect of various culture conditions on the production of the distinctive red-orange pigmentation by *B. linens*. It was found that the addition of 0.2% sodium oxalate to the culture medium resulted in a definite increase in color production. Added sodium oxalate chelates all soluble calcium salts in the medium, the elimination of which results in the production of a more intense color by pigment-forming bacteria (115). The addition of peptone or casein to the medium also affected color production. Cultures supplemented with 10% peptone or 10% casein produced an intense color; relatively little color was produced on tryptone extract agar alone. Cultures incubated in an atmosphere of oxygen showed more intense color production than did those incubated in air.

The color reaction of *B. linens* with various chemical reagents has been used as a taxonomic tool for differentiating *B. linens* from other species (49). Spot testing of *B. linens* with NaOH, KOH, LiOH, glacial acetic acid, aniline, H_3PO_4, perchloric acid, H_2SO_4, and HCl was studied. Of particular interest was the reaction of *B. linens* with NaOH, which produces a unique carmine red color, and the reaction with acetic acid, which produces a unique salmon pink color. On the basis of these color reactions, *B. linens* could be distinguished from other microorganisms possessing yellow-orange pigmentation such as *Staph. aureus*, *Staphylococcus epidermidis*, *Micrococcus flavus*, *Micrococcus citreus*, *Mycobacterium phlei*, and *Sarcina lutea*. However, Grecz and Dack (49) did not study yellow coryneform bacteria, which are frequently confused with *B. linens*. In a similar study involving the reaction of *B. linens* with various reagents, Jones et al. (68) included species from the genera *Arthrobacter*, *Corynebacterium*, *Nocardia*, and *Mycobacterium*. Of the 93 strains tested, only 5 gave the characteristic color reactions reported by Grecz and Dack (49) to be typical of *B. linens*. Two were culture collection strains of *B. linens* (ATCC 9174 and NCIB 8546), but the other 3 were *Corynebacterium* spp. (2 strains) and *Arthrobacter* sp. (1 strain), indicating that, although spot testing with various chemicals is a good method for separating *B. linens* from other species, it is not exclusively selective for *B. linens*. The authors also tentatively suggested, based

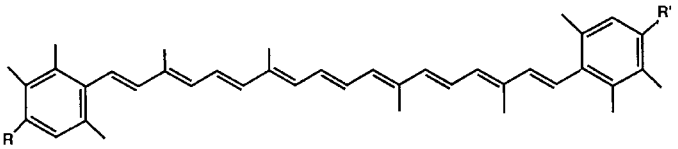

Figure 1. Chemical structure of the aromatic pigments produced by *Brevibacterium linens*. R, R′ = H; isorenieratene, R = H, R′ = OH; 3-hydroxyiso-renieratene, R, R′ = OH; 3,3′-dihydroxy-isorenieratene (79).

on spectroscopy analysis of hot methanol extracts of *B. linens*, that the pigment produced by *B. linens* was a carotenoid-like compound, which was located in the membrane fraction of the cell; the cell-wall fraction was unpigmented.

Kohl et al. (79) positively identified the pigments of *B. linens* as aromatic carotenoids using mass spectroscopy. It was found that *B. linens* produces three pigments; the main pigment was identified as the aromatic carotenoid, 3,3′-dihydroxy-isorenieratene, and the two other pigments were identified as isorenieratene and 3-hydroxy-iso-renieratene (Figure 1). The dihydroxyphenyl carotenoid is responsible for the color shift from yellow-orange to pink-purple when *B. linens* colonies are treated with an alkaline solution. The effect of light on pigment production by *B. linens* strains has been studied (94). To determine the effect of light on pigmentation, a comparison was made between growth and pigment formation in the dark and light by 82 strains of coryneform bacteria (including *B. linens*). Sunlight had a pronounced effect on pigmentation of a number of *B. linens* isolates from cheese and sea fish. Some isolates were white when grown in the dark but were orange when grown in light. Some strains of *B. linens* developed orange pigmentation in both dark and light conditions, indicating heterogeneity within the species. It was noted that light exerted its effect during the growth of the microorganisms; cultures grown in the dark did not turn orange to any significant extent when exposed to light. It was also suggested that, in addition to light, oxygen and certain nutritional conditions may affect pigment production by *B. linens* and coryneforms.

Font de Valdez et al. (46) studied the bacterial microflora of Roquefort cheese; a total of 32 strains were isolated, 12 of which were orange pigment producers under both light and dark conditions, and 8 strains produced an orange pigment only in the presence of light. The remaining isolates were grey-white. No pigment production was observed at pH 6.0 or below in the absence of NaCl, and the highest color intensity was registered at pH 7.0 in the presence of NaCl. Morphological and physiological studies on the orange-pigmented strains indicated that most of them were closely related to *B. linens*.

Ferchichi et al. (37) observed that the concentration of dissolved oxygen affected pigment production by *B. linens* cultured in a fermentor. An intense orange color was obtained after 20 h at 50 or 70% saturation; at 25% saturation, the same color intensity was not reached until 38 h. At 12.5% saturation, the intense orange color did not appear. The addition of L-methionine to cultures also appeared to affect pigment production; pigmentation appeared about 2 h after the addition of L-methionine to a basic medium, and maximal pigmentation occurred 25 h after this addition.

The pigments of *B. linens*, isorenieratene and its hydroxy derivatives, are unusual natural compounds and are one of the main distinctive characteristics of the organism. Isorenieratene was isolated originally from *Mycobacterium leprae* (later identified as *M. phlei*) and has been found only in several other strains of *Mycobacterium*; in the phototrophic bacterium, *Phaeobium*, a brown *Chlorobium species*; in *Corynebacterium fascians*, and in *Streptomyces mediolani*. The two hydroxy derivatives of isorenieratene found in *B. linens* have an even more restrictive distribution in nature, and *Strep. mediolani* is the only other organism in which they have been also been identified (79). This uniqueness and restricted distribution of isorenieratene and its hydroxy derivatives makes them useful chemosystematic markers; all *B. linens* strains tested produced these unique pigments.

MISCELLANEOUS BIOCHEMICAL AND PHYSIOLOGICAL PROPERTIES

Peptidogylcan

The primary function of the bacterial cell wall is to maintain the integrity and rigidity of the cell. The major component of the cell wall of Gram-positive bacteria, such as *B. linens*, is peptidoglycan. The peptidoglycan found in *B. linens* is type A1γ (111). The amino group of the D-asymmetric carbon of the meso-diaminopimelic acid forms a peptide bond with the carboxyl group of D-alanine of an adjacent peptide subunit. No interpeptide bridge is involved; this kind of crosslinkage is known as direct crosslinkage. In addition to *B. linens*, peptidoglycan type A1γ is found in numerous species of the genera *Brevibacterium*, *Corynebacterium*, and *Arthrobacter* and in *L. monocytogenes*. Differences in peptidoglycan type and

structure between microorganisms can be used for taxonomic discrimination. However, the analysis of peptidoglycan is expensive and laborious.

Insecticide Degradation

The degradation of 1,1,1,-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) by two strains of *B. linens* (ATCC 9172 and ATCC 9175) has been demonstrated in media augmented with these insecticides (83). Strain ATCC 9175 degraded the insecticides to a greater extent than did strain ATCC 9172; both strains degraded 1,1,1,-trichloro-2,2-bis(*p*-chlorophenyl)ethane more extensively than 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene. *Geotrichum candidum*, which is present on smear surface-ripened cheeses, also has the ability to degrade these insecticides.

Antibiotic Resistance

In a study on the resistance of various dairy microorganisms to a range of 30 different antibiotics, *B. linens* was found to be reasonably resistant (105). The various dairy microorganisms included in this study could be listed in order of decreasing resistance to the antibiotics tested: *Leuconostoc dextranicum, B. linens, Streptococcus faecalis, Streptococcus durans, Lactobacillus bulgaricus* (now *Lactobacillus bulgaricus* ssp. *delbrueckii*), *Streptococcus lactis* (now *Lactococcus lactis* ssp. *lactis*), *Streptococcus diacetylactis* (now citrate-utilizing *Lactococcus lactis* ssp. *lactis*), *Streptococcus thermophilus, Streptococcus cremoris* (now *Lactococcus lactis* ssp. *cremoris*), *Staph. aureus, Staph. epidermidis,* and *Micrococus varians*. The antibiotics to which *B. linens* was found to be resistant were methicillin, nafcillin, cloxacillin, oxacillin, furadantin, and nalidixic acid.

Fermentation Studies

Because *B. linens* is required commercially in the dairy industry for the production of smear surface-ripened cheeses, the fermentation technology involved in the cultivation of the microorganism is important from the perspective of the commercial producers. Such industrial producers of *B. linens* cultures include Chr. Hansen A/S (Hørsholm, Denmark), Wiesby (Niebüll, Germany), Texel (Dangé Saint Romain, France), Gist-brocades (Delft, Netherlands), and SBI-SKW (La Ferté Sous Jouarre, France). In the industrial production of *B. linens*, it is important for economic reasons to have an efficient process. High oxygenation rates, cultivation temperature, and medium ingredients are significant factors affecting the final cost of these products to the industrial producer.

Famelart et al. (34) studied the effect of temperature, pH, and dissolved oxygen on the growth of *B. linens* ATCC 9175 in a fermentor. It was found that maximum growth occurred when the dissolved oxygen was maintained at 50% saturation; growth was inhibited when the pO_2 was at 40 or 60%. Famelart et al. (35) studied the metabolism of *B. linens* in a fermentor on a basal medium supplemented with amino acids, sodium L-lactate, NaCl, and vitamins. Biomass yields indicated that amino acids are the limiting factor for *B. linens*. The most rapidly consumed amino acids were tyrosine, phenylalanine, arginine, proline, glutamate, and histidine and appeared to be the limiting substrates. The degradation of arginine in ripening cheeses was reported (112) to occur via the production of citrulline, ornithine, and putrescine. Tokita and Hosono (121) noted the production of large quantities of ornithine by *B. linens* when oxygen concentration was low. Ammonia was also produced, but large quantities were produced only when the medium contained amino acids alone or in a large proportion compared with the carbon source.

Potential Biotechnological Applications

In addition to the commercial production of *B. linens* for use as a cheese-ripening agent by the previously mentioned culture companies, a number of patents have been published involving the use of *B. linens* in biotechnological applications. They are mostly Japanese patents, which describe the use of *B. linens* in waste water treatment, vitamin production, and pharmaceutical processes. It is not known whether any of these patents are used commercially. These biotechnological applications are summarized in Table 3.

USE AS A CHEESE RIPENING ACCELERANT

One of the most significant challenges facing the dairy and food industry in the area of cheese manufacture is the length of the ripening period for certain cheese varieties. For Cheddar cheese, a minimum of 6 mo of ripening is normally necessary; the cost of storage during this time using controlled temperature and atmosphere is significant (US$ 1/tonne/d; P. F. Fox, 1997, unpublished observation). Several different approaches have been used to accelerate ripening: 1) elevated ripening temperature, 2)

TABLE 3. Potential biotechnological applications of *Brevibacterium linens*.

Company	Reaction	Application	Reference
Allied Colloids Ltd,[1] Bradford, United Kingdom.	Reaction of amidase with polyacrylamide gel particles	Production of polyacrylamide with reduced monomer impurities	(36)
Mitsui Toatsu Chem Inc.,[2] Tokyo, Japan.	Conversion of 3-amino-1-propanol to 3-amino-1-propionic acid	Vitamin production (3-amino-1-propionic acid precursor for pantothenic acid)	(3)
NOF Corporation,[2] Tokyo, Japan.	Production of antimutagenic agent	Inhibition of mutagens in fats and oils	(6)
Seiko Kakoki,[2] Japan.	Degradation of acetone	Organic waste-gas or waste water treatment	(7)
Taki Kagaku Kk,[2] Hyogo, Japan.	Conversion of soybean milk serum to biomass without organic acid production	Waste disposal	(5)
Tanabe Seiyaku Co.,[2] Singapore.	Conversion of 1-(4-methoxyphenyl)-2-amino propanone to 1-(4-methoxyphenyl)-2-amino propane	1-(4-methoxyphenyl)-2-aminopropane possess bronchodilator activity	(4)

[1]European patent.

[2]Japanese patent.

manufacture from raw milk, 3) manufacture with genetically modified organisms, 4) addition of adjunct cultures, 5) addition of exogenous enzymes, and 6) addition of flavor precursors. The adoption of any of these techniques requires that the ripening time be reduced without leading to any negative flavor or textural characteristics.

The use of *B. linens* as a cheese-ripening accelerant can be either direct or indirect. The direct use of the microorganisms involves the addition of viable cells or cell extracts to the curd or cheese milk. Indirect methods involve the addition of the extracellular proteinase or extracellular aminopeptidase to the cheese milk; some success has been reported with both methods; however, some caution is necessary in the interpretation of these results due to the difficulty associated with the accuracy and reliability of the various taste panels employed in these studies.

Chen et al. (19) used lyophilized extracts from several cheese-related microorganisms, including *B. linens*, to accelerate the ripening of low fat Cheddar. The lyophilized extracts, prepared by grinding the cells, were added to the low fat Cheddar curd after milling. The cheeses were then assayed monthly for flavor (no details of taste panel) and for proteolysis up to 6 mo. At the end of the ripening period, the treated cheese showed significantly higher phosphotungstic acid-soluble nitrogen and TCA-soluble nitrogen than did the control. Bitterness and other off-flavors were not detected, but the treated cheese did not exhibit pronounced Cheddar cheese flavor. An intracellular cell-free extract from *B. linens* has been used individually and in combination with Neutrase (a commercial neutral proteinase, Novo Enzyme Products Ltd., Windsor, United Kingdom), to accelerate the ripening of Ras cheese (32). During ripening, the cheeses treated with either Neutrase alone or with the cell-free extract had a significantly more intense Ras cheese flavor but were more bitter at the end of ripening (as judged by a trained taste panel).

Hayashi et al. (54) used the partially purified extracellular proteinases produced by *B. linens* to accelerate the ripening of Cheddar cheese. Cheddar cheese was manufactured in the conventional way with the addition of the proteinase to the milled curd. After ripening for 2 mo at 12°C, the cheeses were assessed for flavor, bitterness, and proteolysis. The flavor scores (judged by a trained taste panel of 17 persons) for the treated cheeses were increased significantly without significant amounts of bitterness. The TCA-soluble nitrogen increased in the treated cheeses, and β-CN was degraded extensively. In a further study by Hayashi et al. (55), a partially purified extracellular aminopeptidase from *B. linens*, combined with a commercial neutral proteinase, was used to accelerate Cheddar cheese ripening. After ripening for 2 and 3 mo at 12°C, the flavor intensity (judged by a trained taste panel of 24 persons) of the enzyme-treated cheese had increased significantly compared with the untreated control; no significant bitterness was detected. Protein hydrolysis to peptides and amino acids, as measured by TCA-soluble and sulfosalicylic acid-soluble nitrogen, was significantly increased compared with the untreated control. Significant differences in casein degradation, as measured by urea-PAGE, between the control and treated cheese were also observed. Hayashi et al. (55) suggested that the ripening period for Cheddar could be reduced from 4-6 mo to 2 mo using this system.

Brezina et al. (17) accelerated the ripening of Dutch-type cheese (variety not specified) by the separate and combined addition of extracellular proteinase and extracellular aminopeptidase from *B. linens*. There was a very good correlation between the amount of proteinase added and water-soluble nitrogen; unfortunately, there was also a good correlation between the amount of proteinase added and bitterness (judged by a trained taste panel of 15 persons). However, bitterness was successfully reduced in cheeses manufactured using a combination of the extracellular proteinase and the extracellular aminopeptidase, and the ripening process was accelerated.

The proteinase of *B. linens* has also been used to accelerate flavor development in Ras cheese slurries (89). Addition of the proteinase to the slurry increased the ratio of soluble nitrogen to total nitrogen, the ratio of nonprotein nitrogen to total nitrogen, soluble tyrosine, and tryptophan as incubation progressed. Pronounced flavor development (no details of taste panel) was noticed in the proteinase-treated slurry. In a study involving the use of *B. linens* as a debittering agent, Brezina et al. (17) found that it was possible to eliminate the bitterness of peptides almost completely by using an intracellular aminopeptidase preparation from a strain of *B. linens*. A process for the prevention of bitterness in Camembert cheese using a mutant of *B. linens* was also developed.

One of the obvious limitations of employing the extracellular proteinase or aminopeptidases from *B. linens* is that a large amount of purified or partially purified enzyme is required for the treatment of small quantities of curd. However, ample scope remains for increasing the proteolytic capacity of *B. linens* by variation in medium composition, different cultivation conditions, and the use of genetically modified organisms.

Kim and Olson (76, 78) evaluated the production of methanethiol and hydrogen sulfide in milk fat-coated microcapsules containing *B. linens* and methionine or cysteine for possible flavor development of Cheddar cheese. Production of methanethiol from methionine occurred aerobically and anaerobically, but the production was three- to fourfold greater under aerobic conditions; optimum pH and temperature for methanethiol production was 8.0 and 26°C, respectively. Production of hydrogen disulfide from cysteine required anaerobic conditions (77). The interactive effect of methanethiol and hydrogen sulfide production was also examined (75), in which the two substrates, cysteine and methionine, were coencapsulated with *B. linens* or encapsulated separately with *B. linens* and mixed together. The production of hydrogen sulfide from cysteine in microcapsules was slightly reduced by the methanethiol produced from methionine. However, the presence of hydrogen sulfide stabilized the methanethiol produced from methionine in milkfat-coated microcapsules and, consequently, increased the level of methanethiol. It was concluded that this microencapsulation system is capable of producing adequate amounts of methanethiol for the development of Cheddar cheese flavor, but no cheese trials were performed to support this claim.

Recently, *B. linens* cultures have been used as adjunct cultures to accelerate the flavor development of Cheddar cheese (B. Weimer, 1997, personal communication). The addition of *B. linens* to the cheese had a very positive effect with a significant reduction in the ripening time required to produce a mature flavor in Cheddar cheese. The cultures were also effective at increasing the flavor intensity of low fat Cheddar cheese.

CONCLUSIONS

A large number of questions remain to be answered in many areas of the physiology, metabolism, genetics, and taxonomy of *B. linens*. Although the commercial importance of *B. linens* is small compared with that of the lactic acid bacteria, it is nevertheless an important dairy microorganism. The precise role of *B. linens* in relation to smear surface-ripened cheese will remain difficult to define until more knowledge is available about its metabolite production and enzymatic activities.

Most research to date on *B. linens* has demonstrated that the physiological and metabolic activities of the bacterium are significantly strain dependent. The reported differences in the biochemical properties and the number of its extracellular proteinases is a clear example; differences are also reported for its aminopeptidase, esterase, and lipase activities. Further strain variation is evident by differences between the biochemical properties and target microorganisms of the bacteriocins produced by *B. linens*. The heterogeneity among different stains of *B. linens* has also been confirmed by studies of DNA-DNA homology (40), which indicate those strains at present designated as *B. linens* constitute at least two distinct groups. This degree of strain variation within the species *B. linens* must be addressed by taxonomists to facilitate further research of strains currently classified as *B. linens*.

In the area of enzymology, several areas of future research of *B. linens* are of particular interest. The purification and characterization of further peptidases and esterases would improve the understanding of the role of *B. linens* in the ripening of smear surface cheeses. Furthermore, the purification of the various amino acid converting enzymes would be of benefit. In the area of carbohydrate metabolism, the metabolic pathways and enzymes involved have not yet been established in *B. linens*. There is a paucity of information on most aspects of the genetics of *B. linens*. It would be of interest to identify and characterize the genes that code for the extracellular proteinase and aminopeptidase, bacteriocins, L-methionine-γ-demethiolase, carotenoid production, and antibiotic resistance.

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