Examination of *Brevibacterium linens* by an Electrophoretic Zymogram Technique

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SUMMARY

Fifteen isolates of *Brevibacterium linens* were tested by polyacrylamide gel electrophoresis. Protein patterns and zymograms of 18 intracellular enzyme activities (α -naphthyl acetate and β -naphthyl butyrate esterases, tributyrinase, alcohol-, lactate-, malate-, succinate-, glucose-6-phosphate-, 6-phosphogluconate-, isocitrate-, alanine- and glutamate-dehydrogenases, tetrazolium oxidase, catalase, peroxidase, arylamidase, proteinase and amylase) and four extracellular enzyme activities (α -naphthyl acetate esterase, arylamidase, proteinase and amylase) were examined. Enzyme activities were demonstrated by either specific staining reactions or by the subsequent diffusion of the proteins into a substrate layer. Striking variations of the enzyme patterns occurred among the strains. The variation, however, depended largely on the activity under consideration. Eight different enzymes were selected to calculate a matrix of similarity. The results indicated a separation of the strains into two major subdivisions or biotypes. Only one strain (BL107) differed markedly from the others.

INTRODUCTION

The taxonomic status of *Brevibacterium linens*, technologically important for cheese ripening, has received comparatively little attention. When characterized by the morphological, physiological and biochemical criteria as listed in *Bergey's Manual of Determinative Bacteriology* (Breed, Murray & Smith, 1957) there are difficulties in differentiating *Brevibacterium linens* from several representatives of the genus *Arthrobacter* or *Corynebacterium* (Mulder *et al.* 1966; Da Silva & Holt, 1965; Bousfield, 1972).

Since the usual biochemical routine tests do not discriminate between homologous and analogous metabolic pathways and frequently represent only a fraction of the genome, they fail to express the true genetic relationships between micro-organisms.

Grecz & Dack (1961) tried to identify the pigmented strains of *Brevibacterium linens* by means of colour reactions. Friedman, Nelson & Wood (1953) and Brandl & Petutschnig (1972) described great differences in the proteolytic and lipolytic capacities of strains of *B. linens*.

The determination of the electrophoretic patterns of various enzyme activities (zymogram technique) which detect minor structural differences within the proteins has made a valuable contribution to microbial taxonomy (Garber & Rippon, 1968; Baptist, Shaw & Mandel, 1969). Similar electrophoretic investigations have proved the usefulness of esterases (Morichi, Sharpe & Reiter, 1968; El Sharkawy & Huisingh, 1971), catalases (Cann & Willox, 1965; Robinson, 1966) and various dehydrogenases (Bowman, Brubaker, Frischer & Carson, 1967; Baptist, Shaw & Mandel, 1971).

Table 1. *Strains of Brevibacterium linens studied and their sources*

Strain*	Source
BL107	Nederlands Instituut voor Zuivelonderzoek (NIZO), Ede, The Netherlands
ATCC9174	Nederlands Instituut voor Zuivelonderzoek (NIZO), Ede, The Netherlands
ATCC9175	Nederlands Instituut voor Zuivelonderzoek (NIZO), Ede, The Netherlands
LAKTOFLORA208/BMSM/9	Dairy Research Laboratory, Association for the Production of Dairy Cultures, Prague, Czechoslovakia
B	Bundes-Lehr- und Versuchsanstalt für alpenländische Milchwirtschaft, Rotholz, Austria
W	Bundes-Lehr- und Versuchsanstalt für alpenländische Milchwirtschaft, Rotholz, Austria
a	Hochschule für Bodenkultur, Institut für Milchwirtschaft, Vienna, Austria,
BW	Commercial culture, Germany
S	Commercial culture, Austria
A	Isolated from an Austrian cheese
D	Isolated from an Austrian cheese
I	Isolated from a French cheese
2	Isolated from a French cheese
CH/O	Isolated from a Swiss cheese
CH/W	Isolated from a Swiss cheese

* Subsequently, strains BL107, ATCC9174, ATCC9175 and LAKTOFLORA208/BMSM/9 are referred to as 107, 9174, 9175 and 208 respectively.

Enzymatic investigations of *B. linens* established the presence of gelatinase, amylase, peroxidase, xanthine dehydrogenase, catalase and peptidase (Thomasow, 1950) and amino acid decarboxylase (Hosono & Tokita, 1969*a*). Metabolic activities which give rise to volatile compounds from carbohydrates, fats and citric acid (Hosono, 1968*a*; Hosono & Tokita, 1969*b*) and from amino acids (Hosono, 1968*b*) have also been reported.

In the present work an electrophoretic zymogram technique was applied to several strains of *Brevibacterium linens* to ascertain their relationships.

METHODS

Bacteria and growth conditions. The strains (Table 1) had already been clearly characterized as *Brevibacterium linens* (E. Brandl & K. Petutschnig, unpublished) by the usual tests of Breed *et al.* (1957). All were Gram-positive, produced catalase, liquefied gelatin, were lipolytic on tributyrin agar, reduced nitrate, turned litmus milk alkaline and grew in the presence of 15% NaCl. None of these strains produced acid from glucose, acetyl methyl carbinol, H₂S or indol. All produced an orange pigment except strains CH/W, A and ATCC9175.

Cultures maintained on nutrient agar (Difco) slants were used to inoculate 30 ml of nutrient broth plus 0.1% casein according to Hammarsten (Merck, Darmstadt, Germany), incubated for 24 h at 20 °C with vigorous rotary agitation. This culture was used to inoculate another 30 ml volume and the incubation process was repeated. This was then used to inoculate 300 ml of the same medium in 1 l Erlenmeyer flasks and incubated at 20 °C for 48 h with agitation.

Preparation of intracellular enzymes. The growth was harvested by centrifugation at 34 000 g for 20 min at 4 °C, washed twice with physiological saline and finally suspended in distilled water at a concentration of approximately 1 g wet wt/ml and sonicated for 5 min at 1.5 A in an ice bath. Cell debris was removed by centrifugation at 34 000 g at 4 °C for 15 min. The supernatant was frozen immediately using dry ice and then lyophilized. Preparations were stored up to 2 weeks at -20 °C.

Preparation of extracellular enzymes. The supernatant from the first centrifugation was concentrated at 25 °C in a rotary vaporizer 100:1, dialysed overnight against tap water and stored at -20 °C.

Disc-electrophoresis. Polyacrylamide gel electrophoresis was carried out according to the methods described by Maurer (1971). Electrophoresis was performed in the apparatus described by Foissy (1974*a*). Separation gels, containing 11.0 % (w/v) acrylamide were prepared with 0.3 M-tris to 0.06 M-hydrochloride buffer, pH 8.9, in a glass tray of internal dimensions 240 × 150 × 3 mm. The spacer gel consisted of 2.7 % (w/v) of acrylamide and a 0.05 M-tris to 0.06 M-hydrochloride buffer, pH 6.8. The buffer in both the upper and the lower reservoirs was 5 mM-tris to 0.04 M-glycine, pH 8.3. Sample pockets were filled with, respectively, 30 µl of a solution containing 2% intracellular proteins or 50 µl of a concentrate prepared as described for extracellular preparations. In both cases sucrose was added to increase the density of the samples. Temperature was maintained at 4 °C. The initial voltage, 150 V, was increased to 350 V when the buffer front had reached the interface between the spacer and the separation gel. Electrophoresis was discontinued when the buffer front had run 10 cm. To standardize results the relative electrophoretic mobilities of bands were calculated as E_r values (ratio of the distance moved by the protein to the distance moved by the front) (Gottlieb & Hepden, 1966). β -Casein A was used as the standard protein ($E_r = 0.38$).

Localization of enzymes

Esterases. After electrophoresis the gels were stained using the method of Lawrence, Melnick & Weimer (1960).

Tributyrylase. The medium described by Lawrence, Fryer & Reiter (1967) was used as a substrate. Gels were pressed on the substrate surface. Clearing of the tributyrin agar indicated tributyrinase activity.

Dehydrogenases. The methods of Green, Goldberg & Blendon (1967) were used. After electrophoresis the gels were incubated in the dark at 37 °C for approximately 2 h in a reaction mixture containing 15 mg nicotinamide adenine dinucleotide (NAD), 50 mg nitro blue tetrazolium chloride (NBT), 65 mg KCN, 2 mg phenazine methosulphate (PMS) and 100 ml of 0.1 M-tris-hydrochloride buffer, pH 8.5. To this solution, 1.8 g sodium-DL-malate, 2.7 g succinic acid disodium salt, 1.69 g monosodium glutamate, 2 ml sodium lactate (60 % syrup), or 0.9 g alanine were added, depending upon the enzymes being assayed.

Prior to polymerization 0.2 % MgCl_2 was added to the gel solution as a cofactor for alcohol dehydrogenase. The reaction mixture consisted of 15 ml substrate (1.0 ml absolute ethyl alcohol, 3.3 ml 1.0 M-tris, in a total volume of 33 ml distilled water), 9 mg NAD, 4 mg PMS, 20 mg NBT and 100 ml 0.1 M-tris-hydrochloride buffer, pH 8.5.

The methods of Baptist *et al.* (1971) were used to detect glutamate, isocitrate, 6-phosphogluconate and glucose-6-phosphate dehydrogenases; nicotine amide adenine dinucleotide phosphate (NADP) was used instead of NAD as a cosubstrate. The substrates (1.0 M-sodium glutamate, 0.1 M-trisodium isocitrate, 0.5 M-trisodium-6-phosphogluconate and 0.25 M-disodium glucose-6-phosphate, respectively) were dissolved in 0.5 M-tris-hydrochloride buffer, pH 7.1.

Tetrazolium oxidase. Patterns were tested as for the dehydrogenases, but with substrate omitted. Blank bands on the blue background marked areas of activity.

Catalase. The method was based on that of Robinson (1966). Prior to polymerization 1 % starch was added to the acrylamide gel solution. After electrophoresis the gel was immersed in 2 % (v/v) hydrogen peroxide solution, washed thoroughly with running water

and then covered with acidic potassium iodine, 2 % (w/v), for 30 to 60 s. Catalases appeared as white bands against the blue background.

Peroxidase. The method of Schrauwen (1966) was followed using a benzidine–guaiacol–hydrogen peroxide system.

Proteinase. Activity was demonstrated after diffusion of the enzymes from the electropherograms into a gelatin agar layer. Areas of hydrolysis were developed with acidic mercuric chloride (Foissy, 1974*b*).

Arylamidase. Following the method of Smith & Rutenburg (1966) the gels were incubated for 5 h in a solution of L-leucyl- β -naphthyl-amide hydrochloride (0.68 mM) in 0.1 M-phosphate buffer, pH 7.0. The diazonium salt Garnet GBC was used as a coupling agent for the naphthol released.

Amylase. It was necessary to add 3.0 % (w/v) starch to the polyacrylamide gel solution prior to polymerization. After electrophoresis the gels were incubated in a moist atmosphere for 24 h. The unhydrolysed starch was stained with 2 % (w/v) acidic potassium iodine solution. White bands against a blue background showed amylase activity.

RESULTS

Preliminary tests showed that under the conditions chosen all of the enzymes studied were negatively charged. Thus only the anodic gels were examined.

The bacterial extracts were tested on numerous occasions to verify the existence of minor electrophoretic differences. Only bands which were consistently present were noted.

A complex protein pattern was detected in the electropherograms after staining with Coomassie-blue. This made comparison of the various strains difficult.

Intracellular enzymes

Fig. 1, 2 and 3 (*a*, *b*, *c*) are schematic drawings of the patterns of a few intracellular enzyme activities for 15 strains of *Brevibacterium linens*. The strains are listed according to the similarities of their esterase patterns and this system is maintained for all diagrams.

Esterolytic activity against synthetic substrates was found in all strains of *Brevibacterium linens* examined (Fig. 1*a*, *b*). β -Naphthyl butyrate as a substrate showed more esterolytic enzymes than α -naphthyl acetate, as seen in the increased number of faint bands. Except for strain 107, all strains showed strongly stained bands at $E_f = 0.58$ or $E_f = 0.65$. These main esterase bands also reacted with tributyrin.

The dehydrogenases were also multimolecular systems. Seven of the 15 strains (BW, W, 9174, 2, CH/W, CH/O, a) possessed a strong band at $E_f = 0.35$ which was evident when malate, succinate, glucose-6-phosphate or 6-phosphogluconate were used as a substrate. The highest activity occurred in the case of 6-phosphogluconate. Strain 107 was again different as it possessed a double band at $E_f = 0.41$ to 0.43. Despite their heterogeneous general impression, the complex dehydrogenase patterns did reveal analogies between certain strains. However, they were not as good as those based on esterase patterns.

A single band of catalase activity occurred in a small E_f -range with only minor variations, except for strain 1 which possessed two bands.

The tetrazolium oxidase patterns were very similar, band $E_f = 0.45$ occurred in 11 of the strains.

Arylamidase and amylase activities were not detectable in the intracellular preparations.

Fig. 1. Electrophoretic patterns of enzyme activities of some *Brevibacterium linens* strains. Intracellular activities: (a) α -naphthyl acetate esterase; (b) β -naphthyl butyrate esterase; (c) tributyrinase; (d) alcohol dehydrogenase; (e) lactate dehydrogenase; (f) malate dehydrogenase.

Fig. 2. Electrophoretic patterns of enzyme activities of some *Brevibacterium linens* strains. Intracellular activities: (a) succinate dehydrogenase; (b) glucose-6-phosphate- and 6-phosphogluconate dehydrogenases; (c) isocitrate dehydrogenase; (d) alanine dehydrogenase; (e) glutamate dehydrogenase; (f) tetrazolium oxidase.

Fig. 3. Electrophoretic patterns of enzyme activities of some *Brevibacterium linens* strains. Intracellular activities: (a) catalase; (b) peroxidase; (c) proteinase. Extracellular activities: (d) α -naphthyl acetate esterase; (e) arylamidase; (f) proteinase.

Fig. 4. Matrix of similarity coefficients of eight enzymes of *Brevibacterium linens* strains. Data are from Fig. 1 (b, d, e, f), Fig. 2 (a, c, f) and Fig. 3 (a).

Extracellular enzymes

Fig. 3 (d, e, f) summarizes the enzyme activity in the extracellular preparations examined. Distinct and highly reproducible patterns could be detected among the proteinases. The esterase pattern was weak and showed correlations with intracellular main bands. Arylamidase activity was also weak. No amylase activity was detectable.

Determination of similarity

The results of the intracellular activities of β -naphthyl butyrate esterases, alcohol-, lactate-, malate-, succinate- and isocitrate-dehydrogenases, and tetrazolium oxidases and catalases were used as a representative sample to calculate the matrix of similarity shown in Fig. 4. The similarity of each pair of strains was calculated as the percentage of enzymes which appeared to be the same type, out of the total number of enzymes available for comparison with that pair.

DISCUSSION

We wished to see if the electrophoretic differentiation of the enzyme contents of 15 strains of *Brevibacterium linens* could be used as an improved basis for an objective differentiation, because by the usual bacteriological tests all strains appeared identical except that strains ATCC9175, A and CH/W lacked pigmentation.

In view of the large number of proteins resolved by Coomassie-blue staining the determination of enzyme patterns appeared to be a useful criterion for differentiation within the species *Brevibacterium linens*, although it may not always be true that enzymes giving the same banding pattern are identical.

The results indicated a division into two major subgroups based on esterase patterns (strains BW, W, 9174, 2, CH/W, CH/O, a, 208, 9175 and A labelled biotype I, and strains B, S, I and D labelled biotype II, see Fig. 4). Strain 107 is unique because it lacked one of the two esterases present in all the other strains.

On the basis of the presence or absence of the typical dehydrogenase band at $E_f = 0.35$, it was also possible to divide all the strains (except for strain 107) into two groups. This division differed from that based on esterases. Strains 208, 9175 and A, which were in the same esterase group as strains BW, W, 9174, 2, CH/W, CH/O and a, appeared in the same group as strains B, S, I and D because they did not exhibit the main dehydrogenase band at $E_f = 0.35$. However, when a larger number of electrophoretic features were used to compare similarity, strains 208, 9175 and A exhibited a greater resemblance to the group in which they fell on the basis of esterase patterns (Fig. 4).

Additionally, although strain a showed the characteristic dehydrogenase activity at $E_f = 0.35$, it did not reach the level of 75 % similarity with the other strains in the esterase subgroup because of its succinate dehydrogenase, tetrazolium oxidase and catalase patterns.

The results of the matrix of similarity based on enzyme activities indicates that the *Brevibacterium linens* strains examined fall into recognizably different groups. The division, however, depends on the percentage level of similarity chosen as a basis, but this is always so (see Sneath, 1972). Assuming that a similarity of more than 50 % calculated from zymograms suffices for a classification into biotypes, we would include within biotype I strains BW, W, 9174, 2, CH/W, CH/O, a, 208, 9175 and A, and in biotype II strains B, S, I and D (Fig. 4). The results with strain 107 indicate that it belongs to a different subgroup. The establishment of a third biotype would be justified if this particular type could be detected again when testing a greater number of strains. This division corresponds exactly with that according to esterase profiles alone and it is interesting that Norris (1964) demonstrated good correlations between esterase patterns and serological relationships in the genus *Bacillus*.

The extent to which the two biotypes characterized in this study can be differentiated by improved or different physiological and biochemical tests should be investigated. Absence of pigmentation in *Brevibacterium linens* is apparently not so decisive a factor as to justify another species. This is in line with the results of Crombach (1971), who could detect no striking differences in the GC-ratio in a DNA analysis of pigmented and non-pigmented strains.

The extracellular esterase, arylamidase and proteinase patterns appear to be of little taxonomic value but are of interest in cheese ripening. Differences in these features might be the reason for the different proteolytic capacities of various strains, but the production of these enzymes depends greatly on physiological and biochemical conditions.

Mention should be made of the proteinase patterns, which show a high degree of heterogeneity. However, in the case of biotype I especially it seems that the individual profiles are

based primarily on the ability of each strain to produce one or other enzyme isoactivity from a probable group-specific pattern of proteinases or, to be more specific, gelatinases. Relations between individual extracellular proteinase patterns and their technological applicability are at present being studied.

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