The Use of Polyacrylamide Gel Electrophoresis in Taxonomy of *Brucella*

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(Received 19 December 1972)

SUMMARY

Polyacrylamide gel electrophoresis was used to compare bacterial proteins of *Brucella abortus*, *B. melitensis* and *B. suis* and of the relatively new members of the genus, *B. neotomae*, *B. canis* and *B. ovis*. With the exception of the two representatives of a new fifth biotype of *B. suis*, all the protein patterns were identical. *Yersinia enterocolitica* IX and *Y. enterocolitica* NCTC10461 gave indistinguishable protein patterns which were distinct from those of *Brucella*. The results suggest that *B. neotomae*, *B. canis* and *B. ovis* are assigned to the correct genus but indicate that the taxonomic status of the *B. suis* biotype 5 representatives should be re-examined.

INTRODUCTION

In systematic bacteriology, techniques which compare the bacterial genome appear to be the most accurate in determining genetic similarity among bacteria (e.g. Hill, 1966; Heberlein, Deley & Tijtgat, 1967). Recently, DNA homology studies have been successfully applied to the taxonomy of *Brucella* (Hoyer & McCullough, 1968*a, b*) but such studies require specialized equipment and are very time-consuming. Since protein structure is genetically determined it is often possible to compare regions of bacterial DNA indirectly by comparing the electrophoretic patterns of bacterial proteins. Thus, electrophoresis of proteins has proved a convenient alternative to direct nucleic acid studies in the classification of organisms such as *Streptomyces* (Gottlieb & Hepden, 1966), *Mycoplasma* (Razin & Rottem, 1967), microaerophilic vibrios (Morris & Park, 1970) and *Mycobacterium* (Haas, Davidson & Sacks, 1972).

In the present study the protein electrophoretograms of members of the three classical species of *Brucella* (namely *Brucella abortus*, *B. melitensis* and *B. suis*) were examined together with the relatively new members of the genus, *B. neotomae*, *B. canis* and *B. ovis*. *Yersinia enterocolitica* IX, which cross-reacts serologically with *Brucella*, and *Y. enterocolitica* NCTC10461 were also studied. The protein patterns obtained were of value in determining the similarity between recognized members of the genus and allowed the taxonomic status of two representatives of a new fifth biotype of *B. suis* (Renoux & Philippon, 1969) to be assessed.

METHODS

Bacteria. With the exception of two strains designated *Brucella suis* biotype 5, which were donated by Dr A. Philippon as freeze-dried cultures labelled B58 and 4607, all the bacteria were from the culture collection maintained at this laboratory (Table 1). The 36 *Brucella* cultures from the laboratory collection had been previously characterized by the methods

Table 1. *Bacteria examined*

Species	Biotype	Strain
<i>Brucella abortus</i>	1	544, 819, 45/20, 899
	2	808/67, 1045/67
	3	3/68
	4	126/68, 240/68
	5	96/67
	6	26/64, 1295/67
	7	63/294, 63/76
	8	ND
	9	30/68, 237/68
<i>B. melitensis</i>	1	16M, REV1, 23/65, 72/65, 83/65
	2	9/63, 114/63
	3	323/63, 43/65
<i>B. suis</i>	1	1330, 187/63
	2	258/63, 266/63
	3	P4/11, P8/3/66
	4	207/63, 220/68
	5*	B58, 4607
<i>B. canis</i>	—	RM6-66
<i>B. neotomae</i>	—	5K33
<i>B. ovis</i>	—	ST63/97
<i>Yersinia enterocolitica</i>	—	Serotype IX, NCTC10461

ND = not done.

* Provided by Dr A. Philippon, Institut National de la Recherche Agronomique, Station de Pathologie de la Reproduction, Nouzilly, France.

recommended by the Subcommittee on Taxonomy of *Brucella* (Stableforth & Jones, 1963; Jones, 1967) and included the type culture of each species examined. The strains were representatives of every biotype of *Brucella* maintained at this laboratory. Bacteria were cultured on serum dextrose agar or trypticase soya agar.

Protein extraction. Cells were harvested from two or more slopes using cold phosphate-buffered saline (100 mM, pH 7.0) and washed once in the same diluent. Protein was extracted by emulsifying 100 mg of bacteria in 100 μ l of phenol + acetic acid + water (4:2:1 parts by weight). The digest was centrifuged at 40000 *g* for 30 min and the supernatant fluid retained. The extracts, which contained the acid + phenol soluble (APS) proteins, were stored at -20°C unless they were required immediately.

Electrophoresis. Protein samples were separated in columns containing 7.5% (w/v) acrylamide, 5 M-urea and 35% (v/v) aqueous acetic acid. These were prepared by adding 12 ml of stock solution A (acrylamide, 7.5 g; urea, 30 g; *N,N'*-methylenebisacrylamide, 0.2 g; aqueous acetic acid (35%, v/v, to 100 ml) to 10 mg of ammonium persulphate. The solution was degassed at 160 Torr for 5 min, the vacuum released and 0.06 ml of *N,N,N',N'*-tetramethylethylenediamine added. This working solution was dispensed into glass running tubes (5 mm internal diam. $\times$ 75 mm length) to a height of 62 mm, overlaid with a few drops of water and allowed to polymerize at 37°C for 90 min. The gels were positioned vertically in the tank of an Analytical Polyacrylamide Electrophoresis Apparatus (Shandon Scientific Co. Ltd, London N.W.10) with 10% (v/v) aqueous acetic acid as the reservoir fluid. Between 50 μ l and 75 μ l of the APS protein sample were layered directly on to the gel through the surface of the acetic acid. (Previous experiments had shown that when APS protein fractions were prepared under standard conditions this volume contained 250 to 300 μ g of protein). The upper electrode served as the anode and a constant

Fig. 1. Electrophoretic patterns of cell proteins from the type cultures of (a) *Brucella abortus* (544); (b) *B. melitensis* (16M), (c) *B. suis* biotype 1 (1330), (d) *B. neotomae* (5K33), (e) *B. ovis* (ST63/97), (f) *B. canis* (RM6-66).

current equivalent to 3 mA/gel was delivered from a Vokam Type 2541 Power Pack (Shandon). After 15 min the current was increased to 5 mA/gel for a further 60 min. A reference gel, reserved for the APS protein sample from *Brucella abortus* s99, was included in every separation. The separated proteins were visualized by immersing the gels in 1.0% Amido Black (prepared in 7% (v/v) aqueous acetic acid) for 30 min and removing unbound stain by washing in 30% (v/v) aqueous acetic acid. In every experiment the protein patterns were compared with the pattern of the reference gel included in that experiment and not with gels from different experiments.

RESULTS

When separating APS proteins it was found to be extremely important to employ standard conditions at all times and only to compare protein patterns separated during the same experiment. When these points were observed the protein pattern of a strain was a constant feature which was usually reproducible. Even so, slight variation in the number of the minor bands was occasionally observed, but whenever this occurred repeat experiments using fresh extracts always produced the expected patterns. A large, heavily staining area was often observed near the bottom of the gels but this was not a true protein band as the strain was only present at the gel surface. (The artefact is seen in Fig. 1 a, for example, but is absent in Fig. 1 f.) Different growth media did not affect the protein patterns and there was no

Fig. 2. Electrophoretic patterns of cell proteins of (a) *Brucella suis* biotype 5 (B58), (b) *B. suis* biotype 5 (4607), (c) *B. suis* biotype 1 (1330).

difference between the electrophoretograms of proteins from rough and smooth organisms. Similarly, the age of the culture did not affect the protein patterns.

The electrophoretic patterns of proteins from all the biotypes of *Brucella abortus* examined (biotype 8 was not available), all the biotypes of *B. melitensis*, *B. suis* biotypes 1, 2, 3 and 4, *B. neotomae*, *B. ovis* and *B. canis* were identical to each other. The patterns obtained from the type culture of each of the six species of *Brucella* are shown in Fig. 1. The protein patterns of strain B58 and strain 4607, the two representatives of *B. suis* biotype 5, were indistinguishable and, although they showed some similarity to those of the other brucellas, these two strains could easily be distinguished from the established members of the genus (Fig. 2). *Yersinia enterocolitica* IX and *Y. enterocolitica* NCTC10461 gave identical protein patterns which were distinct from those of the established *Brucella* species (Fig. 3) and of the two representatives of *B. suis* biotype 5.

DISCUSSION

From the similarity in the protein patterns of all the *Brucella* species (with the exception of *Brucella suis* biotype 5) it is apparent that the patterns obtained from polyacrylamide gel electrophoresis of the proteins reflect the overall relationship between the organisms classified as *Brucella*. *Yersinia enterocolitica* IX shows complete cross-agglutination with *Brucella* and tests with monospecific antiserum have shown that the cross-reacting antigen of *Y. enterocolitica* IX contains determinants of similar specificity to the A determinants of *B. abortus*

Fig. 3. Electrophoretic patterns of cell proteins of (a) *Yersinia enterocolitica* IX, (b) *Y. enterocolitica* NCTC 10461, (c) *Brucella* sp. (ST63/97).

(Corbel & Cullen, 1970). *Yersinia enterocolitica* NCTC 10461 does not cross-react serologically with *Brucella*. The protein electrophoretograms of the two *Yersinia* strains were identical but distinct from those produced from the *Brucella* cultures. Thus, the technique appears to differentiate at the generic level despite intergeneric serological cross-reaction.

The results indicate that *Brucella neotomae* (Stoenner & Lackman, 1957) is closely related to the three classical species and therefore that the organism has been assigned to the correct genus. Similarly, although the generic rank of *B. ovis* has been questioned in the past (e.g. Meyer & Cameron, 1956; Jones, 1967) the results presented here suggest that the organism is a member of the genus *Brucella*. Diaz, Jones & Wilson (1967) using serological techniques and Hoyer & McCullough (1968*a*) using DNA homology techniques both reached similar conclusions. Recently a new member of the genus, *B. canis*, was described by Carmichael & Bruner (1968). The DNA homology studies of Hoyer & McCullough (1968*b*) and the more conventional studies of Jones, Zanardi, Leong & Wilson (1968) showed that the inclusion of this organism in the genus *Brucella* was justified. Again, bacterial protein electrophoretograms afford independent confirmation that the isolates of Carmichael & Bruner (1968) are *Brucella*.

Strains B58 and 4607, isolated from cattle and sheep respectively, were two representatives of isolates claimed by Renoux & Philippon (1969) to differ from the other biotypes of *Brucella suis* in their resistance to Safranin O and in their oxidative metabolic patterns. These workers therefore proposed that their isolates be accepted as representatives of a new fifth biotype of *B. suis* (Renoux & Philippon, 1969). Having demonstrated that the 36 *Brucella* cultures from the culture collection at this laboratory produced identical protein electro-

phoretograms, it was immediately evident from the protein patterns of B58 and 4607 that these two organisms were not *Brucella*.

Clearly the taxonomic status of *Brucella suis* biotype 5 should be re-examined.

Polyacrylamide gel electrophoresis is a technique which should only be used for taxonomic purposes with great care as reproducibility can be difficult to attain and difficulties may arise in making objective comparisons between different gel patterns. Despite these problems, well-marked electrophoretic differences have been demonstrated both at the intergeneric level (Sacks, Haas & Razin, 1969) and at the interspecies level (Lund, 1965; Razin, 1968) using polyacrylamide gel electrophoresis. A degree of selectivity can be introduced into the technique by considering specific enzymes rather than a total protein mixture (e.g. Norris & Burges, 1963; Norris, 1964). This not only eases the problem of comparison between gels but often has the effect of increasing the sensitivity of the technique (Morris, 1971). In the present work, studies on enzymes may have proved useful in distinguishing between species of *Brucella*. However, the preparation of extracts with enzymatic activity would necessitate not only a larger quantity of bacteria but also an extra step in the procedure to break the cells. These are important considerations when working with pathogens. The use of whole-cell digests has the advantage of requiring only a small quantity of bacteria from which the protein can be extracted in one simple step. This, together with the demonstration that the electrophoretic properties of the proteins were not affected by colony dissociation, age or cultural conditions, suggests that the technique may be particularly useful in assigning new and unusual isolates to the genus *Brucella*.

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