Proteinase Isoenzyme Patterns of *Bacteroides nodosus*: Distinction between Ovine Virulent Isolates, Ovine Benign Isolates and Bovine Isolates

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Bacteroides nodosus isolates from ovine virulent footrot and ovine benign footrot and bovine isolates of low virulence for sheep were distinguishable from each other by their proteinase isoenzyme patterns after polyacrylamide gel electrophoresis. Variants of low virulence were not always distinguishable from their virulent parent strains. The molecular weights of the isoenzymes ranged from 70000 to 129000. The relationship of isoenzyme patterns to virulence is discussed.

INTRODUCTION

Bacteroides nodosus has been isolated from cases of ovine virulent footrot (Beveridge, 1941), ovine benign footrot (Thomas, 1962; Egerton & Parsonson, 1969) and interdigital dermatitis in cattle (Laing & Egerton, 1978). The latter two isolates do not cause virulent footrot in artificially challenged sheep, and therefore efforts to eradicate and prevent spread of these strains have been given low priority in virulent footrot control programmes in Australia (Thomas, 1962; Egerton & Parsonson, 1969; Stewart, 1979). To assist these programmes, diagnostic tests based on proteinase characteristics have been developed to differentiate *B. nodosus* from cases of virulent and benign footrot (Thomas, 1962; Egerton & Parsonson, 1969; Depiazzi & Richards, 1979; Stewart, 1979). These tests do not distinguish between ovine benign isolates and bovine isolates, and it is not known whether cattle and sheep have their own specific strains of *B. nodosus*. It is also not known whether differences in proteinase characteristics observed in the diagnostic tests result from quantitative or qualitative enzyme differences.

This paper describes a method for producing proteinase zymograms which differentiate the two types of ovine isolates and the cattle isolates from each other, and shows that the various isolates have both quantitative and qualitative differences in their proteinase enzymes. An investigation is also made of a suggestion that benign *B. nodosus* isolates arise from virulent isolates.

METHODS

Bacterial strains and growth conditions. *Bacteroides nodosus* strains 65, 80, 91, 135, 137 and 144 were isolated from clinical cases of ovine footrot in New Zealand. Strains 141 and 142 were isolated from interdigital dermatitis in New Zealand cattle. Strain ATCC 25549, an ovine virulent isolate, was obtained from the American Type Culture Collection. Strains A198 from virulent footrot and A134, A178 and A305 from benign footrot were isolated from Australian sheep and were obtained from the McMaster Laboratory, Division of Animal Health, CSIRO, Sydney, Australia. The prefix A is used to distinguish them from New Zealand strains. *Bacteroides nodosus* variants 91M, 91C and A198M were derived from serially passaged liquid cultures of wild-type strains 91

and A198 as described by Skerman *et al.* (1981). The virulence characteristics of these strains and culture variants in artificial challenge experiments have been described previously (Skerman *et al.*, 1981).

The organisms were grown anaerobically at 37 °C in liquid Trypticase/arginine/serine medium (Skerman, 1975) to maximum cell density ($1-3 \times 10^8$ cells ml⁻¹) at the end of the exponential phase of growth.

Preparation of proteinase zymogram. Bacteria were removed from cultures by centrifugation and the supernatants were concentrated with Carbowax to give sufficient proteinase in 10 µl of each sample to produce a clear reaction in the zymogram. The number of bacteria from which each extracellular proteinase sample was derived is shown in Fig. 1. Samples (10 µl) were applied to a horizontal slab polyacrylamide gel for electrophoresis as described by Every (1981*a*). Electrophoresis in 7% (w/v) acrylamide gel was for 5 h at 1000 V and 15–35 mA, and proteinase isoenzymes were detected by a gelatin-in-agar overlay method (Every, 1981*b*). The overlay consisted of a 0.7 mm thick agar film (1.5%, w/v) containing 0.5% (w/v) gelatin (Difco), 3 mM-NaN₃, 1 mM-NiCl₂ and 20 mM-Tris/HNO₃ buffer, pH 8.8. The overlay was incubated in contact with the polyacrylamide gel slab for 25 min at 37 °C. Zones of gelatin clearing in the agar film were made visible by precipitation of the unhydrolysed gelatin with saturated ammonium sulphate solution. The zymogram was photographed against a black background which gave the isoenzyme bands a dark appearance.

Molecular weight determination. Molecular weights of proteinase isoenzymes were calculated by the method of Slater (1969) using 9–17% (w/v) gradient polyacrylamide gels, and the standard proteins from Pharmacia electrophoresis calibration kits, as described by Every (1981*a*). Electrophoresis of samples and standard proteins was for 16 h at 600 V and 7–25 mA. The isoenzymes were located directly in the polyacrylamide gel electrophoretogram by the following procedure. Whatman 3MM paper saturated with 50 mM-Tris/HNO₃ buffer (pH 8.8) containing Hammarsten casein (BDH; 10 mg ml⁻¹) was placed on the area in the gel containing separated proteinase isoenzymes. After incubation for 20 min at 37 °C the gel was stained with Coomassie Blue R250 (Wyckoff *et al.*, 1977) to give a negative-staining effect: clear areas corresponding to isoenzyme bands appeared against a blue background of unhydrolysed stained casein.

RESULTS AND DISCUSSION

The results (Fig. 1 and Table 1) showed that all the *B. nodosus* strains and variants secreted a mixture of proteinase isoenzymes of various molecular sizes and that the ovine virulent isolates, ovine benign isolates and bovine isolates could be distinguished from each

Table 1. *Molecular weights of B. nodosus proteinase isoenzymes*

Isoenzyme number				10 ⁻³ × Molecular weight
Ovine virulent isolates	Ovine benign isolates	Bovine isolate 141	Bovine isolate 142	
•	•	•	B'1	70
Ov1	Ob1	B1	•	75
•	•	B2	•	77
Ov2	•	•	•	78
Ov3	•	B3	•	82
•	Ob2	•	•	83
Ov4	Ob3	B4	B'2	88
•	Ob4	B5	•	ND
•	Ob5	•	B'3	92
Ov5	•	B6	•	94
Ov6	•	•	•	97
•	•	B7	•	98
•	Ob6	•	B'4	101
•	Ob7	•	•	ND
Ov7	•	•	•	107
•	•	B8	•	110
•	Ob8	•	B'5	114
Ov8	•	•	•	123
•	Ob9	•	B'6	129

ND, Not determined. Although isoenzymes Ob7 and B5 cannot be seen in Fig. 1, they were seen in trace amounts in the original zymograms and are known to be present at higher concentrations in the supernatants of early-exponential phase cultures (unpublished results).

Fig. 1. Proteinase isoenzyme patterns after electrophoresis of culture supernatants of various *B. nodosus* clinical isolates and culture variants in 7% polyacrylamide gel. The numbers of the strains and variants are shown at the top of the zymogram with, in parentheses, the number of bacteria ($\times 10^{-7}$) from which each extracellular proteinase sample was derived. The ovine virulent strain A198 has been included at both sides of the zymogram to make it easier to compare isoenzyme patterns. By extracting individual isoenzymes from the 7% polyacrylamide gel and re-running them on gradient gels, it was shown that the relative positions of isoenzymes in the two gel systems were the same. Thus, the isoenzyme number system used in this figure is the same as that in Table 1.

other by their proteinase isoenzyme patterns. The isoenzymes of all the virulent isolates had the same characteristic molecular weight distribution, but each isolate had different quantities of individual isoenzymes; this was especially noticeable with strain 91. Other virulent isolates from New Zealand sheep (strains 80, 135, 137 and 144), not shown in Fig. 1, had similar isoenzyme patterns. Only two isoenzymes (Ov1 and Ov4) of the virulent isolates had the same molecular weights as isoenzymes (Ob1 and Ob3) of the ovine benign isolates (Table 1). All the ovine benign isolates had similar isoenzyme molecular weight distributions, but they differed in the quantity of each isoenzyme. In contrast, the isoenzymes of the two bovine isolates 141 and 142 differed in molecular weight and in relative concentration (Table 1). Bovine isolate 142 had five isoenzymes (B'2, B'3, B'4, B'5 and B'6) in common with ovine benign isolates, one isoenzyme (B'2) in common with virulent isolates, one novel isoenzyme (B'1) and only one isoenzyme (B'2) in common with bovine isolate 141. Bovine isolate 141 had four isoenzymes (B1, B3, B4 and B6) in common with virulent isolates, three isoenzymes (B1, B4 and B5) in common with ovine benign isolates, and possibly three novel isoenzymes (B2, B7 and B8).

Since Beveridge (1941) first suggested that *B. nodosus* proteinase may be associated with pathogenesis of footrot in sheep, there have been several reports of a correlation between the virulence of *B. nodosus* and proteolytic activity on casein (Thomas, 1962; Egerton & Parsonson, 1969), hide powder-azure (Depiazzi & Richards, 1979) and elastin (Stewart, 1979; Skerman *et al.*, 1981). The presence of low levels or the absence of certain Ov-type isoenzymes (Table 1) in the ovine benign isolates and bovine isolates may account for their low elastase activity (Stewart, 1979; Skerman *et al.*, 1981) and the rapid loss of hide powder-azure degrading activity in stationary phase cultures (Depiazzi & Richards, 1979; Stewart, 1979) in comparison to virulent isolates. This suggestion is currently being examined by comparative substrate specificity and enzyme stability studies using purified individual isoenzymes.

Variants 91M and A198M have been shown to have low virulence characteristics similar to the ovine benign isolates (Skerman *et al.*, 1981) and it was considered that the benign isolates might have arisen from virulent isolates in an analogous way to the production of culture variants from their parent virulent strains. In this case the M-variants might be expected to have similar proteinase isoenzyme patterns to the benign isolates. In fact, the M-variants had electrophoretically similar isoenzymes to their parent virulent strains, and although variant 91M had greatly reduced amounts of isoenzymes, the qualitative and quantitative isoenzyme distribution of variant A198M was almost identical to that of its parent virulent strain (Fig. 1). Hence, the *in vitro* changes in *B. nodosus* appear not to be relevant to conditions *in vivo*, and an alteration in other virulence factor(s), such as pili (Skerman *et al.*, 1981), might account for the low pathogenicity of these variants. Variant 91C, which had no pathogenic properties at all, had very small amounts of isoenzymes (Fig. 1) and virtually no pili (Skerman *et al.*, 1981). However, the isoenzymes that were detectable in variant 91C were identical in molecular size to some of the isoenzymes in its virulent parent strain. It is not known whether the production of culture variants has a genetic basis, but the demonstration that the various clinical isolates produce different molecular forms of isoenzymes from each other shows that they are genetically distinct.

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