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Differentiation of *Achromobacter*-like strains from human blood by DNA restriction endonuclease digest and ribosomal RNA gene probe patterns

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SUMMARY

Variation amongst *Achromobacter*-like strains was examined by DNA restriction endonuclease digestion and rDNA gene patterns generated using a non-radioactive probe. Chromosomal DNA was extracted from 12 cultures representing *Achromobacter* groups B, E and F, all from human blood cultures. DNA fingerprinting using *Eco*RI, *Hae*III or *Hin*dIII sub-divided the strains in a similar manner to that obtained by their protein patterns. The *Hae*III patterns, with their small number of bands, were the easiest to interpret. The *Eco*RI patterns included a species-specific triplet of bands but minor band patterns allowed further differentiation. The *Achromobacter* group F strains comprised a separate taxon and were distinct from the group B and E strains by all techniques examined. The study demonstrates that, in addition to total DNA digest analysis, rDNA gene restriction patterns provide a simple but discriminatory electrophoretic method for distinguishing within *Achromobacter* groups B and E.

INTRODUCTION

Traditionally, clinical microbiologists regarded *Achromobacter* as peritrichously flagellated saccharolytic organisms and *Alcaligenes* as peritrichously flagellated non-saccharolytic forms. Although *Achromobacter* was omitted from the Approved Lists of Bacterial Names [1] it was subsequently revived [2] with *Achromobacter xylosoxidans* as the type and only species. When this species and *Alcaligenes denitrificans* were found to be closely related both were reclassified in *Alcaligenes* as *A. xylosoxidans* subspecies *xylosoxidans* and *A. xylosoxidans* subspecies *denitrificans*, respectively [3]. *Achromobacter* thus has official standing in nomenclature but is currently devoid of any species.

A cluster analysis of phenotypic characters was carried out on *Achromobacter*-like strains [4]. The strains fell into six clusters, *Achromobacter* groups A to F. Group A contained reference strains of *Achromobacter* species biotypes 1 and 2 [5] which have more recently been referred to as Group Vd [6]. Subsequently it was established that group A, plus the minor groups C and D associated with it, represented separate biotypes of a single phylogenetically distinct organism, *Ochrobactrum anthropi* [7].

The taxonomic relationship of *Achromobacter* groups B, E and F remained undetermined. As the strains were isolated almost exclusively from human blood, further study of the group was desirable. Recently, high resolution polyacrylamide gel electrophoresis (PAGE) of proteins with computerized analysis of patterns was used to investigate the interrelationships between these groups [8]. It was evident that the group F strains were clearly quite different from those of groups B and E, whose patterns were very similar. The group B strains could be divided into three sub-phenons (1a–c) and although the group E strains were clustered in one of these sub-phenons (1a) they were nonetheless distinguishable by a visual inspection of the protein patterns.

The aim of this study was to investigate the use of DNA fingerprinting by comparison of total digest patterns and Southern blot hybridization band patterns obtained with a ribosomal (r) RNA cistron probe (rDNA gene patterns [9]). Several restriction enzymes were employed in order to determine their efficacy in differentiating the strains at different levels. The strains studied were chosen to represent both the phenotypic groups (B, E and F [4]) and the phenons/sub-phenons established by SDS-PAGE [8], thereby facilitating a comparison of the results obtained by the various methods.

MATERIAL AND METHODS

Bacterial strains and growth conditions

The 12 isolates used in this study are listed in Table 1, with their reference numbers and sources. All were isolated from the blood of patients in the United Kingdom; two were associated with pyrexia.

All isolates were grown on nutrient agar containing: Nutrient Broth No. 2 (Oxoid: CM67), 25 g/l; New Zealand agar, 12 g/l, for 24 h at 37 °C.

Chromosomal DNA extraction

Chromosomal DNA was extracted and rapidly purified using the guanidium thiocyanate method [10]. The concentration and purity of the DNA sample was determined by absorbance readings at 230, 260 and 280 nm.

DNA digestion and electrophoresis

The DNA (8 μg) was digested with the restriction endonucleases *Eco*RI, *Hin*dIII and *Hae*III (*ca* 1 unit/μg DNA) for 3 h at 37 °C in the buffer recommended by the manufacturers (Northumbria Biologicals Ltd., Cramlington, Northumbria). The digested DNA (12 samples each containing 2 μg DNA) was electrophoresed at 25 V for 16 h in a horizontal 0·7% w/v agarose (Gibco-BRL Ltd: ultrapure, electrophoresis grade) gel in a buffer containing 89 mM Tris, 89 mM boric acid and 2 mM disodium EDTA (pH 8·3). After electrophoresis, the gels were stained in ethidium bromide (1 μg/ml) and photographed for a permanent record.

Preparation of biotinylated probe cDNA

The biotinylated cDNA probe was prepared from 1 μg *Escherichia coli* 16+23S rRNA (BDH Ltd, Poole, UK) using Moloney mouse leukaemia virus reverse transcriptase (Gibco-BRL Ltd), and was biotinylated by the incorporation of

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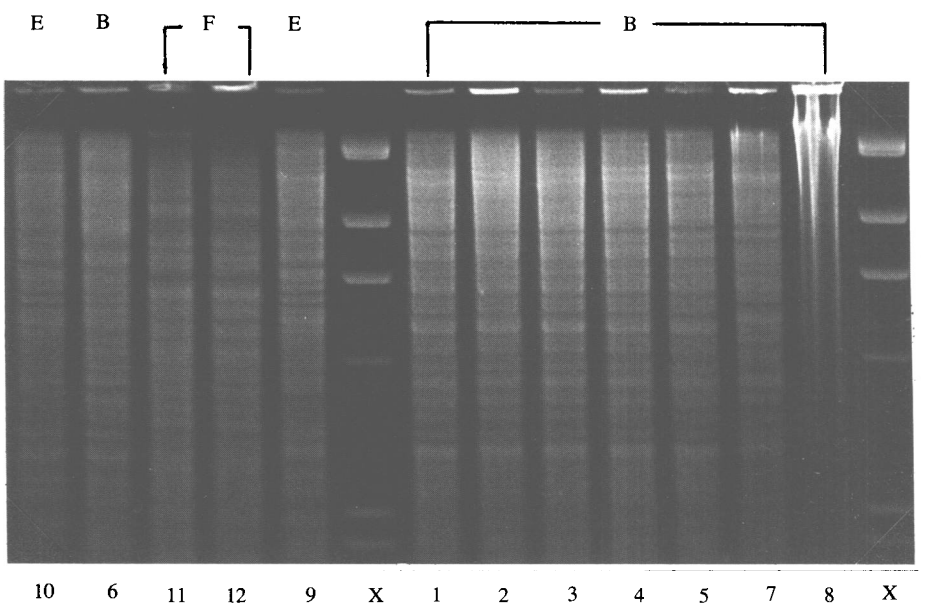
Table 1. *Sources of* Achromobacter-*like strains studied*

Reference no. in figures	Electrophoretic group	Reference no.(s)	Source of isolation: Clinical	Source of isolation: Geographical
Achromobacter group B:				
1	1a	CL26/84	Blood culture; upper respiratory tract infection	Aberdeen, Scotland
2	1a	CL90/88	Blood culture; chest pains, previous streptococcal infective endocarditis, aortic valve replaced	Aylesbury, England
3	1a	CL299/79	One of six sets of blood cultures; probably contaminant	Stoke-on-Trent, England
4	1a	CL43/84	Blood culture	Belfast, Northern Ireland
5	1a	CL43/77† = NCTC 12246	Blood culture; pyrexia of unknown origin	London, England
6	1b	CL622/77† = NCTC 12248	Two separate blood cultures taken on different days; sub-acute bacterial endocarditis	Worthing, England
7	1c	CL137/87	Blood; oncology patient	London, England
8	1c	CL139/87† = NCTC 12249	Blood; oncology patient	London, England
Achromobacter group E:				
9	1a*	CL616/80† = NCTC 12247	Blood culture	London, England
10	1a*	CL731/80	Blood culture	Aberdeen, Scotland
Achromobacter group F:				
11	2	CL45/75	Blood culture	Newcastle-upon-Tyne, England
12	2	CL46/75† = NCTC 12250	Blood culture; elevated temperature *post partum*	Newcastle-upon-Tyne, England

* Outliers of cluster 1a.

† Reference strains of types determined by SDS-PAGE (8).

NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK; all strains prefixed by the letters CL are strains received for identification by the NCTC Identification Services Laboratory.



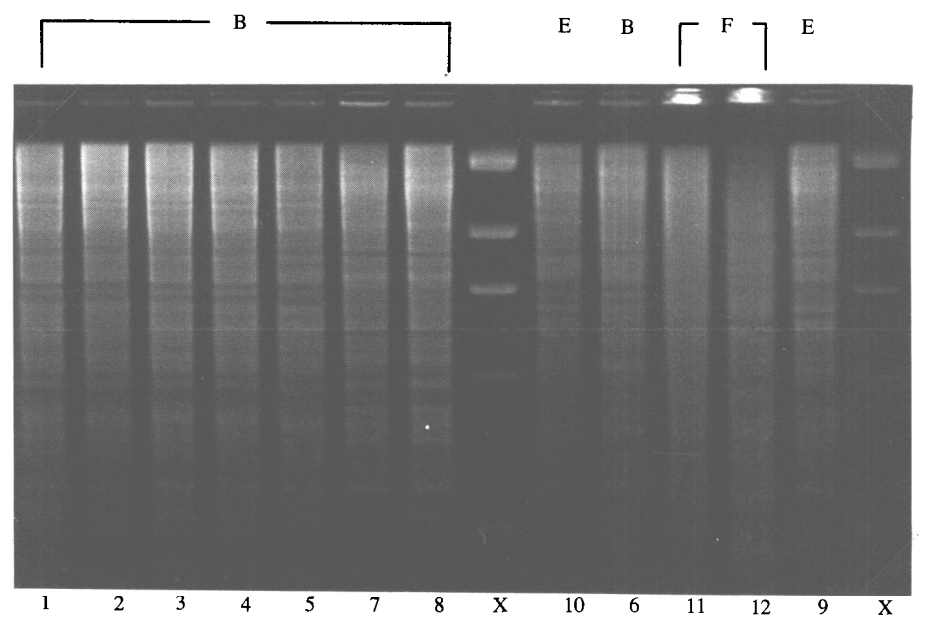


Fig. 1 *a* and *b*. For legend see opposite.

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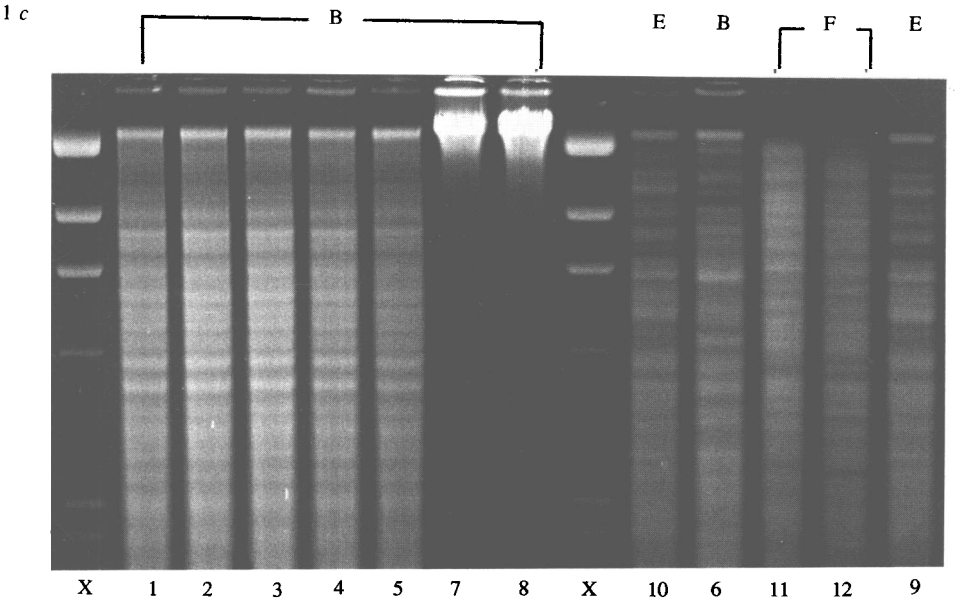

Fig. 1. Agarose gel electrophoretic banding patterns of (a) *Eco*RI, (b) *Hin*dIII and (c) *Hae*III digests of strains of *Achromobacter* group B, E and F chromosomal DNA. The lower numbers refer to strain numbers used in Table 1. Tracks labelled X are the molecular size marker bands (bacteriophage λ DNA digested with *Hin*dIII).

biotin-16-dUTP (Gibco-BRL Ltd) according to previously described methods [11].

Southern blot hybridization

After photography, the DNA in the gel was depurinated by treatment with 0·25 N HCl for 30 min, then denatured in 0·5 M NaOH-1·5 M NaCl for 30 min and neutralized in 0·5 M Tris-HCl-1·5 M NaCl-1 mM disodium EDTA (pH 7.2) for 30 min. DNA was transferred to Hybond-N membranes (0.45 μm pore size: Amersham International) by capillary transfer (18–20 h) or by vacuum-assisted transfer (Vacublot: Anderman & Co. Ltd., Kingston-upon-Thames, Surrey). The membranes were washed once in 2 × SSC (1 × SSC is 0·15 M NaCl plus 0·015 M trisodium citrate), air dried and baked at 80 °C for 2 h. Prehybridization (42 °C for 3–4 h) and hybridization (42 °C for 18 h) were carried out exactly as described previously [12]. The hybridization reactions were visualized colorimetrically with the BluGENE (Gibco-BRL Ltd.) non-radioactive nucleic acid detection system, which contained streptavidin-alkaline phosphatase conjugate and dyes, as recommended by the manufacturer.

Band size estimation

Fragment sizes in the total digest and in the Southern blot hybridization patterns were calculated from migration distances by the DNA SIZE program as described previously [13]. Biotinylated lambda phage (Gibco-BRL Ltd) digested with *Hin*dIII was used to provide the size markers.

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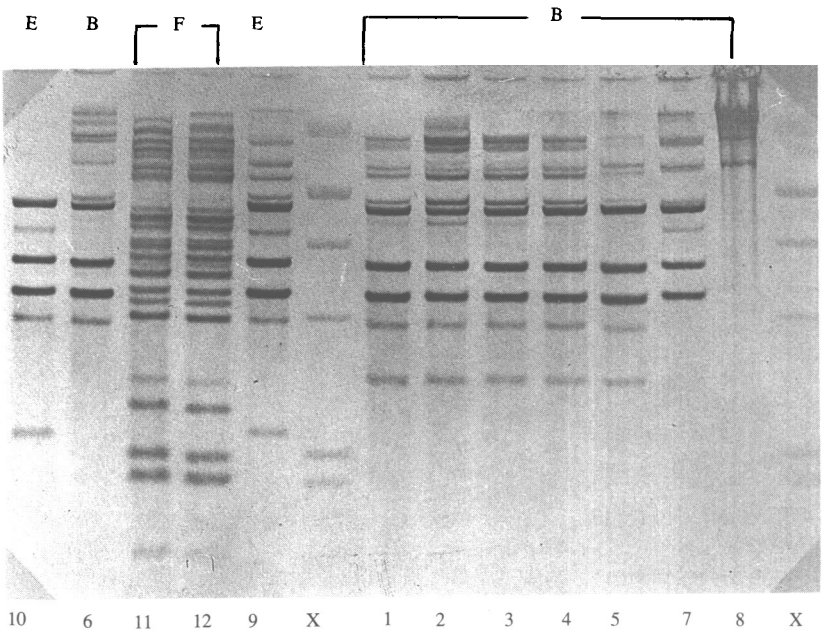
2*a*



2*b*

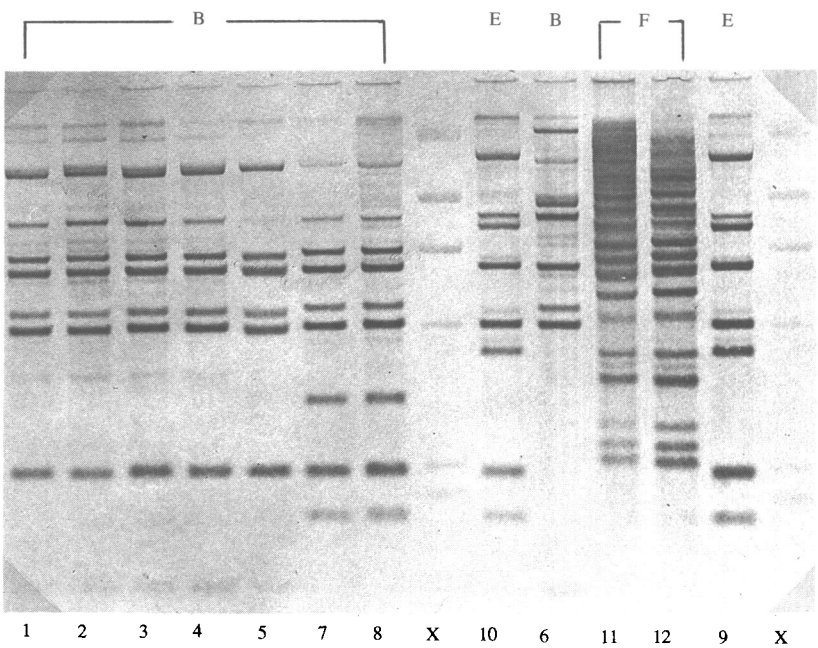


Fig. 2*a* and *b*. For legend see opposite.

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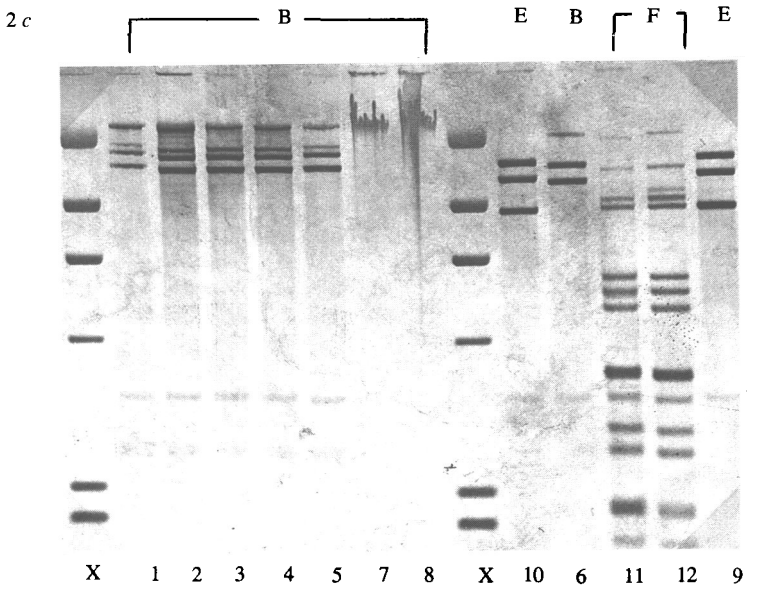

Fig. 2. The rRNA gene patterns for (a) *Eco*RI, (b) *Hin*dIII and (c) *Hae*III digests of strains of *Achromobacter* group B, E and F chromosomal DNA. The lower numbers refer to strain numbers used in Table 1. Tracks labelled X are the molecular size marker bands (bacteriophage λ DNA digested with *Hin*dIII).

RESULTS

Chromosomal DNA digest patterns

Chromosomal DNA samples from eight strains of *Achromobacter* group B and two each of groups E and F were digested with *Eco*RI, *Hin*dIII and *Hae*III which cut DNA from the strains tested with a high frequency to give multiple electrophoretic band patterns (> 15 bands). The fragments with sizes from about 9·4–2·3 kb were generally well resolved (Figs. 1*a–c*). Using all three restriction enzymes, the banding pattern of strains representing groups B, E and F indicated that they were clearly different from each other. Furthermore, the strains of group B could be further differentiated and three pattern types were discernible. The first type comprised five strains (Ref. nos. 1–5, Table 1), the second only one strain (Ref. no. 6) and the third, two strains (Ref. nos. 7 & 8; both strains examined using *Hin*dIII only).

cDNA probing of Eco*RI DNA digests*

The corresponding rDNA gene patterns for the *Eco*RI digests, are shown in Fig. 2*a*. The strains of groups B and E gave less complex, but similar, patterns with 6–11 bands, whereas group F strains gave more complex patterns with 21 discernible bands. All strains produced patterns with bands over the range > 23·1–2·0 kb. The patterns of the group B and E strains were each characterized by a triplet of intense bands, which varied slightly in mobility between groups of strains but were confined to the range 8·4–4·8 kb (see Table 2). The pattern of minor bands found within and below this range reflected differences evident in the corresponding restriction digest patterns; all five protein type 1a strains having

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Table 2. *Estimated sizes of rDNA-containing bands in* Eco*RI*, Hind*III and* Hae*III digests of chromosomal DNA of* Achromobacter *group B, E and F strains*

Achromobacter group		
Biochemical	Protein type	Band Sizes (kb)
*Eco*RI:		
B	1a	20·0, (17·5), (13·0), 12·0, 9·1, **8·4**, (7·9)†, **5·7**, **4·8**, (4·3), (3·1)
B	1b	(33·8), (30·0), (25·0), (24·0), (16·0), (8·6), **8·2**, **5·7**, **4·8**, (4·3)
B	1c	(18·5), (12·5), **8·4**, (7·4), **5·9**, **4·9**
E	1a*	(20·0)†, (14·5)†, (12·0)†, (9·2), **8·4**, (7·2), **5·9**, **4·9**, (4·3), (2·6)
F	2	33·8, 27·4, 21·2, 18·5, 16·3, 15·4, 12·5, (8·2), **7·9**, 7·4, 7·1, 6·8, 6·2, **5·6**, 4·9, 4·6, **4·3**, (3·2), 2·9, 2·3, 2·0
*Hin*dIII:		
B	1a	(33·8), (23·0), **13·8**, 8·2†, (7·1), **6·4**, **5·7**, 4·6, **4·3**, (3·5), 2·2, (1·5)
B	1b	(38·1), 27·4, (15·4), **9·4**, **8·5**, **5·9**, 4·7, **4·3**, (1·5)
B	1c	(33·8), (14·5), (8·5), **6·7**, **5·9**, 4·8 **4·4**, 3·0, 2·2, (1·9)
E	1a*	(38·1), **16·3**, 8·5, **7·8**, **5·9**, **4·3**, **3·8**, 2·2, (1·9)
F	2	**12·3**, **10·3**, **8·7**, 7·8, **6·8**, **6·4**, **5·7**, **5·0**, 4·5, 3·7, 3·3, (2·6), (2·4), (2·3)
*Hae*III:		
B	1a	**31·2**, 19·8, **18·1**, **14·9**, (3·1), (2·6)
B	1b	25·7, **15·4**, **12·6**, (3·1)
B	1c	Not digested
E	1a*	**17·0**, **13·4**, **9·1**, (3·1)
F	2	(24·3), (15·2), (11·8)†, 10·9, 9·4, 5·8, 5·3, 4·8, **3·5**, (3·1), 2·8, 2·6, 2·1, (1·9)

Band size figures in bold type-face are major bands, figures in normal type-face are medium or intermediate size bands and figures in parentheses are weak or minor bands.

* Outlier to protein type 1a.

† Band not present in all strains of group.

two low intensity bands at 4·3 and 3·1 kb, the single protein type 1b strain with only the 4·3 kb band and the protein type 1c strain examined with a band only at 7·4 kb. The group E strains could be distinguished from these types with bands of 7·2, 4·3 and 2·6 kb.

cDNA probing of Hind*III DNA digests*

The rDNA gene patterns for the *Hin*dIII digests, are shown in Fig. 2*b*. The strains of groups B and E gave patterns with 9–12 bands. Compared with the *Eco*RI patterns, a greater degree of heterogeneity was evident in the banding patterns of these groups. However, strains of both groups and of the three protein types recognized in group B all shared some common bands (see Table 2). By

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contrast, group F strains gave more complex but less resolved patterns of at least 14 bands. All strains produced patterns with bands over the range > 23·1–< 2·1 kb. The pattern of bands reflected differences evident in the corresponding restriction digest patterns; all five protein type 1a, the single protein type 1b and the two protein type 1c strains had banding patterns characteristic of their type. The group E strains could be clearly distinguished from these types. The patterns of the strains of protein types 1a and 1c were characterized by a series of four prominent bands, which varied slightly in mobility between groups of strains but were confined to the range 6·7–4·3 kb (see Table 2). Three of the four bands were present in the pattern of the protein type 1b strain but only two in the group E strain patterns. Similar features were evident in the minor band patterns.

cDNA probing of Hae*III DNA digests*

The rDNA gene patterns for the *Hae*III digests, are shown in Fig. 2*c*. The patterns of group B and E strains were relatively simple and characterized by three or four prominent, high molecular size bands (> 23·1–9·1 kb). Those of the group F strains, as with both the *Eco*RI and *Hin*dIII digests, were more complex consisting of 14 bands which were distributed over the 23·1–< 2·1 kb range. The pattern of bands reflected differences evident in the corresponding restriction digest patterns; all five protein type 1a strains having three intense bands at 31·2, 18·1 and 14·9 kb plus a less intense band at 19·8 kb and the single protein type 1b strain with two intense bands at 15·4 and 12·6 kb plus a minor band at 25·7 kb. The group E strains could be distinguished from these types with three intense bands of 17·0, 13·4 and 9·1 kb. The DNA of neither of the protein type 1c strains were digested by this restriction endonuclease.

DISCUSSION

The potential of DNA fingerprints derived from RNA cistrons as a basis for species identification and possibly typing within species is apparent from previous studies of various groups of microorganisms: cyanobacteria [14], eubacteria [15–18], mycoplasmas [19, 20] and yeasts [21]. Given that strains of *Achromobacter* groups B, E and F have been recovered, so far, only from human blood, they may be of clinical importance. The taxonomic interrelationships of these taxa, both at and above the species level, are now better known and here we have investigated the potential of the DNA technique for discriminating between strains.

Preliminary DNA-DNA hybridization results [8] showed that strains of *Achromobacter* groups B and E are members of a single species whereas the strains of group F are genomically quite different organisms. This was also reflected in the SDS-PAGE protein patterns which showed that the group F strains were quite different; the group B/E strains could be subdivided into three types (phenons 1a–c) with the group E strains appearing as outliers within phenon 1a, and having visually different patterns. In the present study, this classification was also closely mirrored in both the DNA restriction digest patterns and rDNA gene patterns.

The *Eco*RI, *Hin*dIII and *Hae*III rDNA gene probe patterns subdivided the strains in the same way as they were sub-divided by their protein patterns. The *Hae*III patterns were the most easily interpreted due to the small number of bands. Least discrimination was evident from the *Eco*RI patterns, which included

a species-specific triplet of bands but where the minor bands allowed further differentiation. The level of discrimination within a taxon is dependent on the restriction enzyme used, as demonstrated in this study. The enzyme giving greatest discrimination for a given species will vary from one species to another [9, 22, 23]. The degree of discrimination achieved will also vary with the species, reflecting genomic variation intrinsic to that particular organism. For example, rDNA gene probe patterns are able to distinguish separate isolates of *Helicobacter pylori* [22], whereas the technique is able to recognize a number of distinct groups within *Providencia stuartii* [23]. In this study, only a small number of variants were recognized among *Acromobacter* group B/E strains and the level of discrimination achieved was no better than that found by whole cell protein electrophoresis [8].

In earlier studies [12, 24], restriction endonuclease digest analysis of chromosomal DNA has proved to be a highly sensitive method of strain identification. Such patterns appear to be highly stable and are apparently unaffected by changes in other characteristics such as colonial morphology [24], and loss of catalase or urease activity [25]. The rDNA probing method gives concordant results with total digest analysis in most cases. The advantage of the rDNA gene patterns is their relative simplicity ($\leqslant 10$ bands) depending on the endonuclease used, which permits comparisons of large numbers of strains. Use of this method is further facilitated by having a non-radioactive probe that can be synthesized from commercially available *E. coli* rRNA, thus avoiding the need to extract rRNA from the organisms under study. These advantages outweigh the possible disadvantages presented by the greater complexity of the technique. Furthermore, the total digest patterns are generally complex and so need to be of extremely high quality to allow meaningful comparisons to be made between strains, with the added possibility that plasmid bands may also further complicate comparisons. Our results on the *Achromobacter* groups indicate that the use of the rDNA gene patterns is an effective method for delineating within groups B/E and of differentiating these from group F and gives discrimination similar to that obtained using comparisons of total DNA digests or whole cell protein pattern analysis. We conclude that rDNA gene fingerprinting is a valuable molecular tool for the characterization of these *Achromobacter* groups.

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