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Antigenic and genetic homogeneity of *Streptococcus uberis* strains from the bovine udder

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SUMMARY

DNA- fingerprints (*Hind* III) of *Streptococcus uberis* field isolates from New York State and Europe showed substantial homogeneity, but were different to those of the type strain of the newly proposed psychrophilic species *S. parauberis*. *S. uberis* strains had major SDS-heat extracted antigens of molecular masses (M_r) < 14, 40–41, 42–43, 59–61, 80–86 and 118–122 kDa following immunoblotting with rabbit hyperimmune sera. Bovine sera and milk reacted with the 40–41 and 118–122 kDa antigens. Variations in the M_r of particular bands were too unevenly distributed to permit formation of subgroups. Although cross reactive, the sizes of the antigens of *S. parauberis* strain NCDO 2020 were substantially different to those of *S. uberis*, the most prominent antigen having a M_r of 50 kDa. The antigenic and genetic data therefore strongly support the introduction of *S. parauberis* as a distinct species. *S. uberis* strains reacted with antiserum to Lancefield groups B, E, G and P, their grouping reactions showing no correlation with DNA and immunoblot fingerprints. Lancefield grouping of *S. uberis* therefore appears to have little value in identification.

INTRODUCTION

Streptococcus uberis has become one of the most important mastitis pathogens in dairy cows causing about 14–20% of all clinical cases [1–5]. The presence of *S. uberis* has been recognized in more than half of the European and North American herds in which approximately 5% of the cows are persistently infected [1, 6, 7]. In addition, control measures, such as post-milking teat distinfection and use of antibiotics, which have worked well in reducing the incidence of *S. agalactiae* mastitis are not effective in controlling *S. uberis* induced mastitis [1, 8, 9].

The species *S. uberis* has been loosely defined by biochemical properties and cultural morphology [10, 11] and is known for its apparent phenotypic diversity. DNA–DNA hybridization has revealed two distinct subtypes with 80–100% homology among members of a given subtype and 45–65% homology between

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them [11, 12]. Strains from Europe of subtypes I and II express lactate dehydrogenase (LDH) type A and C respectively, whereas American strains display LDH type B or D [12].

Recently, comparative analysis of the 16S ribosomal RNA sequences of a *S. uberis* subtype I and a subtype II strain disclosed important differences [13] and have been the basis of a proposal that subtype II be designated *S. parauberis*, a new species with strain NCDO 2020 as the type strain. *S. parauberis* can only be distinguished from *S. uberis* by its ability to grow at 10 °C, but not by cultural morphology or biochemical properties.

In the past, *S. uberis* has been loosely included in Lancefield group E, because approximately 17% of strains precipitated group E antiserum [14]. However, strains have been described that fell into group P (Europe, US), B, C, G and/or U (Europe) [15–17]. At least three, and as many as 11 or more different serological subtypes within the species have been detected by slide agglutination and precipitation tests [18]. A common antigen was demonstrated by rocket-line immunoelectrophoresis, but was not further characterized [19]. Bacteriophage typing of *S. uberis* strains by lytic phages was unsuccessful because of the low susceptibility of most strains [20]. In order to determine genetic, serologic and antigenic features useful for laboratory recognition of *S. uberis* we have studied DNA- and immunoblot fingerprints, protein profiles, and Lancefield grouping reactions of field isolates from the bovine udder in the United States and Europe. The strains were compared with *S. uberis*-type strain ATCC 19436 and *S. parauberis* sp. nov-type strain NCDO 2020 to evaluate these alternative laboratory approaches in distinguishing the two species, and in defining subpopulations of *S. uberis* for epidemiological tracing.

MATERIALS AND METHODS

Bacterial strains and sera

One hundred and twenty randomly selected non-haemolytic streptococcal strains from quarter milk samples of different herds in New York State were supplied by Dr. R. Gonzalez, New York State Mastitis Control Laboratories, NYSCVM. Based on Gram reaction, production of catalase, fermentation of esculin, inulin, mannitol, trehalose, sorbitol, sucrose, sodium hippurate, but not raffinose (36 h at 37 °C), 35 strains identified as *S. uberis* were selected to represent US field strains. In addition, other biochemically similar *S. uberis* strains studied were strain Compton obtained from the AFRC Institute for Animal Health, Compton, Newbury, United Kingdom, strain ATCC 19436, subtype I, obtained from the American type culture collection and Kiel strains 10009, 10100, 10102, 10173, 10223, 52839, 54707 and 320281 from the German streptococcal collection, Institut für Hygiene, Bundesamt für Milchforschung, 23 Kiel 1. European strains 10009 to 10223 were field isolates collected in Germany in 1967. Strains 52839 and 54707 were also isolated from German milk samples in 1974. Strain 320281 was the reference API strain. Unlike *S. parauberis* strain NCDO 2020, all *S. uberis* strains failed to show growth after 5 days incubation at 10 °C in tryptic soy broth (BBL, Cockeyville, MD). *S. parauberis* sp. nov.-type strain NCDO 2020 was obtained from the AFRC Institute for Animal Health, Compton, Newbury, United

Kingdom and utilized esculin, inulin, mannitol, trehalose, sorbitol, sucrose, sodium hippurate and raffinose.

Other streptococcal species studied included *S. agalactiae*, strains DL and 64–149, *S. dysgalactiae*, strain GM2, *S. equi* strain CF32, *S. zooepidemicus*, strains Z320 and W60, *S. equisimilis*, strain Hipkin, *S. bovis*, strain WINT1, *Enterococcus faecalis* strains WINT2 and BRUN1, *S. viridans* (one strain), *S. lactis* subsp. lactis, strains WINT3 and BRUN2, *S. durans* (one strain), *S. zymogenes* (one strain) and *S. canis*, strains DAUT1 and DAUT2. These strains were from the collection at the NYSCVM, Ithaca, NY. *S. pyogenes* type M12 (strain CS24) was supplied by Dr. P. Cleary, University of Minnesota Medical School, Minneapolis, Minnesota, and *S. dysgalactiae* strain SC1 was from Professor H. Blobel, Institut für Bakteriologie, Justus-Liebig-Universität Giessen, FRG. All strains were grown on 1% CNA-agar (BBL. Cockeyville, MD) supplemented with 5% cow blood or in Todd–Hewitt broth supplemented with 0·3% yeast extract.

Thirty-six sera collected in a leptospirosis study from cows in 22 herds were obtained from Dr. S. Shin, Diagnostic Laboratory, NYSCVM. Six milk samples were obtained from the dairy herd of the Mastitis Laboratory, NYSCVM. This herd had a history of *S. uberis* infection. Rabbit antisera to *S. uberis* strains C4 and R2 and to *S. parauberis* NCDO 2020 [19] were provided by Dr N. Norcross, NYSCVM, and Dr K. Jones, Rockefeller University, NY. The antisera were generated by three intravenous injections of formalin-killed suspensions of strains C4 and R2 in the first week followed by weekly injections until the antibody levels were deemed adequate [19]. *S. uberis* strains R2 and C4 were no longer available at the time of this study.

Determination of the Lancefield grouping reaction

Lancefield grouping reactions were determined in a capillary precipitation test [21] using autoclaved extracts of the streptococci [22] and commercially available group-specific antisera to groups B, D, E, G and P (Wellcome Diagnostics, Dartford, UK).

Antigen extraction and gel electrophoresis

Bacterial cells harvested by centrifugation (3000 g for 15 min) from overnight cultures (7 ml) of each strain were washed twice in distilled water and the antigens extracted by heating in 125 ml buffer [8] for 5 min at 95 °C. The boiled cells were then pelleted by centrifugation and 20 ml of the supernatant was immediately applied to a 3 mm thick sodium dodecyl sulphate (SDS; 0·1%) polyacrylamide (10%) gel (PAGE). Components in extracts were separated for 5 h at 50 mA.

Silver staining

Gels were stained using a modified method of Wray and colleagues [23]. The gels were fixed and SDS removed by four washes in 200 ml ethanol (50%) solution over a period of 48 h. They were then rinsed twice (1 min) in distilled water and incubated for 1 h in alkaline silver nitrate solution (0·8 g $AgNO_3$ in 200 ml 0·17 % NH_3 and 9·5 mM-NaOH). Finally, the gels were washed for 8 min in distilled water and the colour reaction developed in 200 ml distilled water containing 0·005% citric acid and 1.85% formalin. The reaction was stopped after approximately

30 min by transfer of the gel into 50 % ethanol solution. Low molecular mass markers (12, 19, 30, 43, 65 and 92 kDa; Bio-Rad, Richmond, CA) and high molecular mass markers (29, 45, 66, 97·4, 116, 205 kDa; Serva, St Louis, MO) were used to calibrate gels.

Immunoblotting

Bands were electrophoretically transferred for 3 h at 1 A to nitrocellulose membranes (0·22 mm porosity; Schleicher and Schuell, Keene, NH) and the membranes blocked by a 30 min incubation in 3 % gelatin solution. Immunoblots [24] were prepared by incubating the sheets for 90 min in bovine sera diluted 1:100 in phosphate buffer, pH 7·2, with 0·1 % Tween 20 (PBS-Tween) or in milk samples diluted 1:10 in PBS-Tween. Following three washings in PBS-Tween the nitrocellulose was incubated for 90 min with horseradish peroxidase-conjugated protein G diluted 1:1000 in PBS-Tween (Zymed, San Francisco, CA). The reaction was then developed after successive washings in PBS-Tween and PBS by incubation in 4-chloro-1-naphthol solution (0·5 mg/ml) containing 0·03 % H_2O_2. In the case of immunoblots made with rabbit antisera to *S. uberis* strains R2 and C4 and to *S. parauberis* NCDO 2020 antibody binding was amplified by incubation in peroxidase-conjugated goat anti-rabbit serum (1:1000; Zymed, San Francisco, CA) prior to incubation with peroxidase-conjugated protein G.

Preparation of genomic DNA

S. uberis and *S. parauberis* strains were grown for 18 h at 37 °C in filter sterilized 3 ml cultures of THB supplemented with 20 U/ml testicular hyaluronidase (Sigma, St. Louis, MO). Bacteria were centrifuged (2000 ***g*** for 20 min), washed in 10 mM-Tris 5 mM ethylene diamine tetra-acetate buffer (EDTA), pH 7·8, resuspended in 400 ml of the same buffer supplemented with 250 U mutanolysin (Sigma, St Louis, MO) and incubated for 2 h at 37 °C to hydrolyse the bacterial cell walls. Twenty millilitres of 10 % SDS and 5 ml of proteinase K (20 mg/ml; Sigma, St. Louis, MO) were then added and the lysate incubated for 1 h at 37 °C. After the addition of 70 ml saturated NaCl solution and 58 ml hexadecyltrimethyl ammonium bromide solution (CTAB, 10 % in 0·7 M-NaCl) the lysate was heated to 65 °C for 10 min. CTAB polysaccharide complexes were removed by chloroform:isoamyl alcohol (24:1; 560 ml to each tube) precipitation followed by 5 min centrifugation at high speed in an Eppendorf centrifuge. The supernatant was reprecipitated in phenol:chloroform:isoamylalcohol (25:24:1; 560 ml) and centrifuged. The DNA was precipitated from the supernatant by the addition of 340 ml ice-cold isopropanol for 2 h at room temperature, hooked on a sealed and bent Pasteur pipette, transferred into 1 ml ice cold ethanol (70 %) and stored at 4 °C overnight. The DNA was transferred into fresh ice-cold ethanol (300 ml), vacuum dried for 10 h in a Speedvac concentrator and resolubilized for 12 h at 4 °C in 100 ml of 10 mM-Tris 1 mM-EDTA, pH 7·8.

Restriction endonuclease digestion and agar gel electrophoresis

DNA from each strain (2–6 ml) was digested for 12 h at 37 °C with 100 U of the restriction endonuclease *Hind* III and 2·5 mg RNase in a total volume of 20 ml Reacti Buffer 2^R (BRL, Gaithersburg, MD). Electrophoresis buffer (3 ml) was

Table 1. *Lancefield grouping reactions of* S. uberis *strains from the bovine udder*

Reactivity with antiserum to	Strains
Group E	ATCC 19436 Cornell 36, 50, 102
Group B	Cornell 81
Group P	Cornell 27, 38, 71, 91, 93 Kiel 10100, 10102, 320281
Group G	Cornell 56, 57
Group E, P	Kiel 52839, Cornell 79
Groups E, G	Cornell 30
Groups B, P	Cornell 68
Groups E, P, B	Kiel 54707
None	Cornell 1, 2, 3, 4, 13, 14, 15, 37, 41, 44, 47, 53, 58, 63, 65, 80, 82, 83, 84, 85, 86 Kiel 10009, 10173, 10223 Compton

added and the digestion stopped by heating the mixture for 10 min at 65 °C. The DNA digests were electrophoresed for 48 h at 25 V and 4 °C on 1 % agar gels (20 × 15 × 0.9 cm) using 0.089 M-Tris-borate 0.002 M-EDTA buffer. Ethidium bromide-stained gels were visualized at 290 nm [25].

RESULTS

Three of the 45 strains of *S. uberis* produced mucoid colonies (strains Kiel 10173, Kiel 320281 and Compton) on blood agar. The remainder produced smooth colonies. Eight *S. uberis* strains precipitated antiserum to Lancefield group E carbohydrate antigen (Table 1). Twelve strains reacted with group P antiserum, three with group G antiserum and three with group B antiserum. No strain reacted with group D antiserum. Twenty-five strains did not react with any of these antisera, while two strains (Cornell 79 and Kiel 52839) reacted with both groups E and P antisera, strain Cornell 30 reacted with groups E and G and strain cornell 68 reacted with groups G and P. Finally, *S. uberis* strain Kiel 54707 reacted with groups E, B and P and *S. parauberis* NCDO 2020 reacted more weakly with group G.

Extracts of the *S. uberis* strains separated by SDS–PAGE and stained with silver showed multiple bands in the molecular mass (M_r) range from 20 to 130 kDa. Generally, major bands were conserved among the strains, but variations were noticed in the band density. Distinct bands in the M_r ranges of 32–38 and 110-130 kDa varied as much as 4 kDa among the strains. No correlation between these variations and Lancefield grouping reactions was found (Fig. 1).

The separated antigens in extracts of all 45 *S. uberis* and of *S. parauberis* NCDO 2020 were transferred electrophoretically to nitrocellulose membranes and reacted with rabbit antisera to *S. uberis* strains R2 and C4 and to *S. parauberis* NCDO 2020. The major bands in *S. uberis* had M_t of < 14, 40–41, 42–43, 59–61, 80–86 and 118–122 kDa. Minor bands of 26, 28–29, 32, 34, 45–47 and 55 kDa were also evident (Figure 2). Variability occurred in the M_r ranges 40–41, 42–43, 59–61, 118–122 kDa of major antigens and in minor antigens (Figs. 2 and 5). The M_r of

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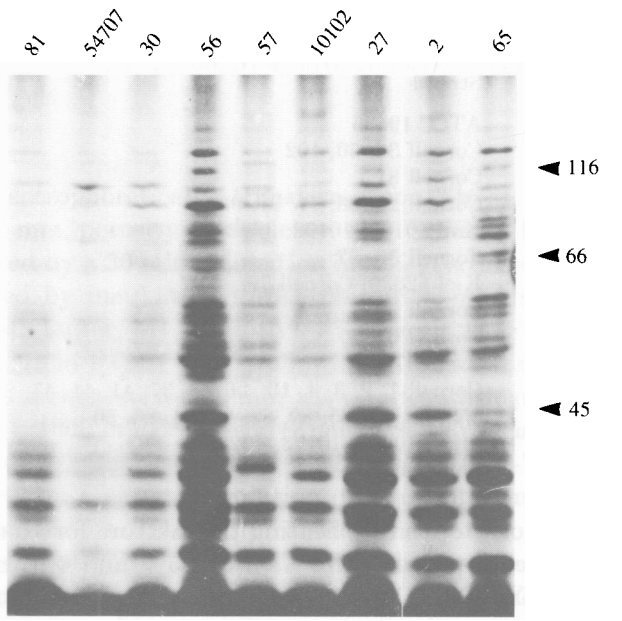

Fig. 1. Silver strain of SDS-heat extracts of *S. uberis* strains Cornell 2, 27, 30, 56, 57, 65 and 81 and Kiel 10102 and 54707 separated on a 10% SDS polyacrylamide gel. Numbers on the right are molecular mass (M_r) standards.

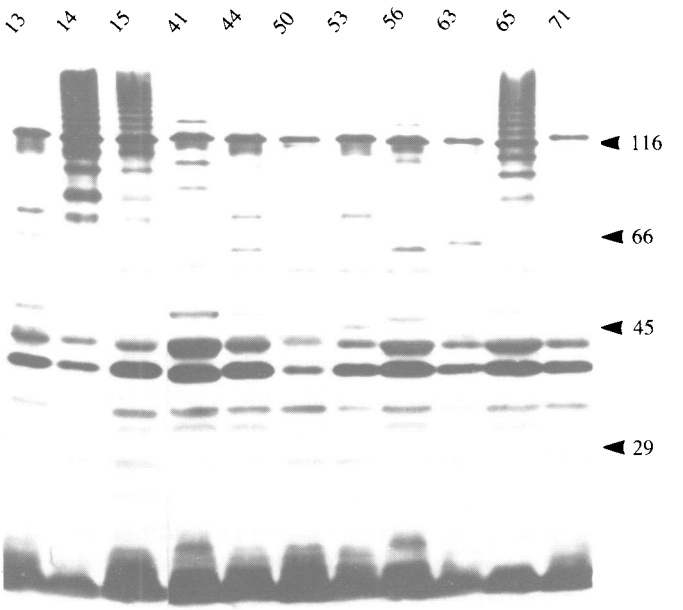

Fig. 2. Immunoblot fingerprints of SDS-heat extracted *S uberis* strains. Extracts of the Cornell strains 13, 14, 15, 41, 44, 50, 53, 56, 63, 65 and 71 were run on a 12% SDS polyacrylamide gel, transferred to nitrocellulose and immunoblotted with rabbit antiserum R2 to *S. uberis* followed by incubation with peroxidase conjugated goat anti-rabbit serum and protein G. The reaction was developed with 4-chloro-1-naphthol. The numbers on the right are M_r standards.

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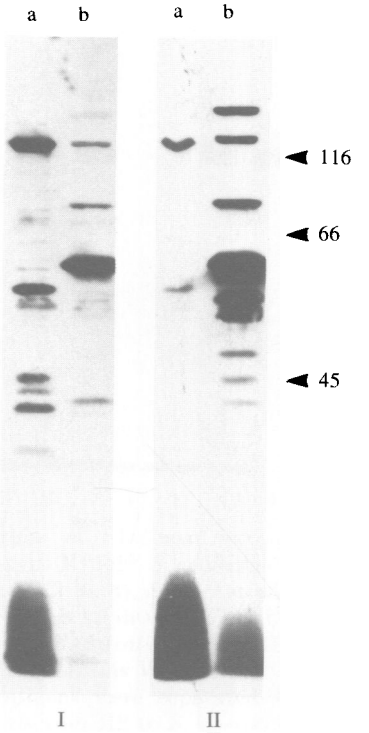

Fig. 3. Immunoblot showing reactivity of SDS-heat extracted antigens of *S. uberis* ATCC 19436 (a) and *S. parauberis* NCDO 2020 (b) with rabbit antisera to *S. uberis* strain R2 (panel I) and *S. parauberis* NCDO 2020 (panel II). Antibody binding was visualized by peroxidase-conjugated protein G and 4-chloro-1-naphthol. The numbers on the right are those of M_r standards.

these antigens were consistent in different extracts of the same strain and were unique to each strain. Therefore, the *S. uberis* stains could not be clustered into subgroups by their antigen profiles. In addition, immunoblot profiles were not correlated with Lancefield groups. *S. parauberis* NCDO 2020 antigens immunoblotted with rabbit antisera R2 and C4 to *S. uberis* exhibited a different pattern reacting with a prominent band at 50 kDa and minor bands at $>$ 120, 78, 42 and $<$ 20 kDa. Rabbit antiserum to strain NCDO 2020 detected *S. parauberis* bands more strongly, but detected bands of *S. uberis* ATCC 19436 only weakly (Fig. 3).

Sera from 17 of 36 cows from 22 different herds reacted strongly with the 41 and 118 kDa antigens in an extract of strain ATCC 19436 (Fig 4, Tracks b, c, e, f, g), but did not detect bands smaller than 25 kDa. Nine sera reacted only weakly (Fig. 4, Tracks a, and f) and ten did not (Fig. 4, Track d). Eighteen strains chosen to include representatives of each Lancefield group or chosen because they gave unique reactions in immunoblot were subsequently tested with a pool of ten of the strongly positive bovine sera (Fig. 5). All sera detected the same bands in each strain suggesting that there were no other unrecognized antigens. Milk samples at a 1 : 10 dilution from 4 of 6 cows from the Cornell mastitis research herd from which *S. uberis* had been repeatedly isolated in the past reacted with the 118 and 41 kDa antigens of strain ATCC 19436.

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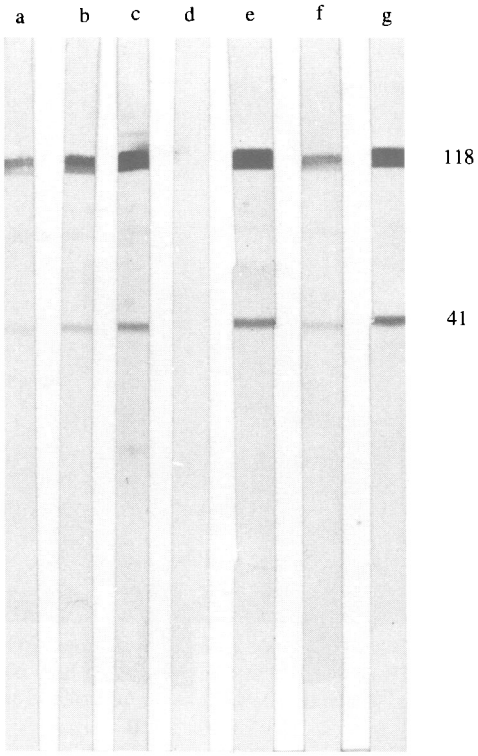

Fig. 4. Immunoblot showing the reactivity of SDS-heat extracted antigens of *S. uberis* strain ATCC 19436 with bovine antisera. Each strip was immunoblotted with a different serum. Antibody binding was visualized by peroxidase conjugated protein G and 4-chloro-1-naphthol. The numbers on the right are the M_r of the major antigens.

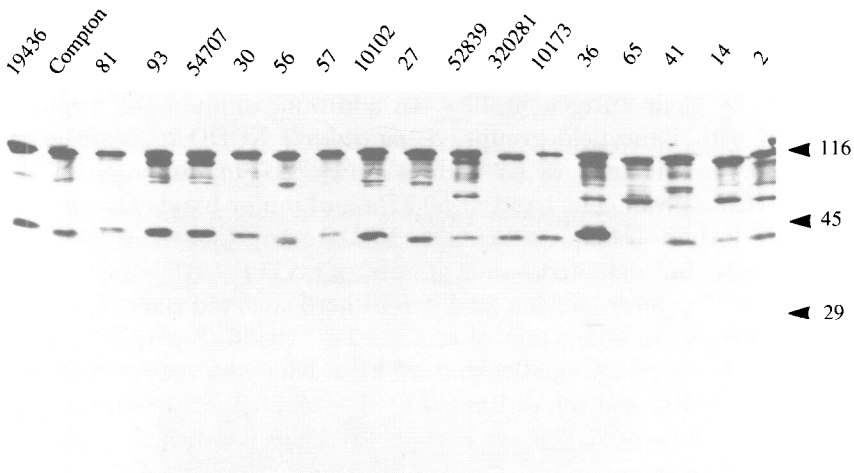

Fig. 5. Immunoblot fingerprints of SDS-heat extracted *S. uberis* strains Compton and ATCC 19436, strains Kiel 320281, 10173, 10102, 54707 and 52839 and Cornell 2, 14, 27, 30, 36, 41, 50, 56, 57, 65 and 81. The extracts were separated on a 10% SDS polyacrylamide gel, transferred to nitrocellulose and reacted with a pool of 10 strongly reactive bovine sera. Binding was visualized by peroxidase-conjugated protein G and 4-chloro-1-naphthol. The numbers on the right are those of the M_r standards.

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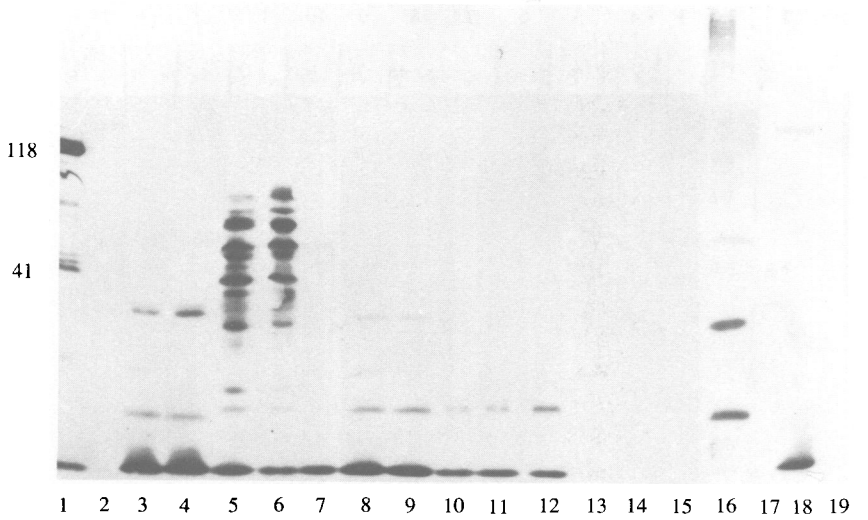

Fig. 6. Immunoblot fingerprints of SDS-heat extracted antigens of *S. uberis* ATCC 19436 (1), *S. pyogenes A*-type M12 (2), *S. agalactiae* strains DL and 64-149 (3, 4), *S. dysgalactiae* strains GM2 and SCl (5, 6), *S. equi* strain CF32 (7), *S. zooepidemicus* strains W60 and Z320 (8, 9), *S. canis* strains DAUT1 and DAUT2 (10, 11) *S. durans* (12), *S. bovis* strain WINT1 (13), *E. faecalis* strains BRUN1 and WINT2 (14, 15), viridans streptococci (16), *S. lactis* strains WINT3 and BRUN2 (17, 18) and one *S. zymogenes* strain (19). The antigens were separated by SDS–PAGE (12%) and immunoblotted with rabbit antiserum R2 to *S. uberis*, followed by incubation with peroxidase-conjugated goat anti-rabbit serum and protein . The reaction was developed with 4-chloro-1-napthol. The M_r of the major antigens of *S. uberis* are shown on the left.

Immunoblot fingerprints of *S. uberis* using rabbit antisera to *S. uberis* strains R2 and C4 were compared with those of *S. pyogenes* A-type M12, *S. agalactiae*, *S. dysgalactiae*, *S. zooepidemicus*, *S. equisimilis*, group G streptococci, *S. fecalis*, *S. bovis*, *S. durans*, *S. lactis* subsp. *lactis* and viridans streptococci. None of these extracts gave a fingerprint profile similar to those of *S. uberis* and, with the exception of *S. dysgalactiae*, only a few antigens of mostly low M_r were detected (Fig. 6). These reactions, especially those of *S. dysgalactiae*, could have been due to non-specific Fc-receptor binding. However, preabsorption of rabbit antiserum R2 with cells of *S. pyogenes A*, *S. dysgalactiae*, *S. equi*, *S. equisimilis*, *S. canis*, but not those of *S. agalactiae*, *S. fecalis* and *S. lactis* subsp. *lactis* strongly reduced or abolished the reactivity of the antiserum with major SDS-heat extracted antigens of *S. uberis*. In this experiment, Fc-receptor activities were blocked by treatment of the streptococcal cells with an excess of *S. uberis* negative rabbit serum prior to incubation in the antiserum (Fig. 7).

DNA fingerprinting performed with the restriction endonuclease *Hin*d III on a cluster of 18 *S. uberis* strains revealed generally similar DNA fingerprints (Fig. 8*a*). However, each strain showed at least two differences in minor bands. No correlation of these differences with Lancefield grouping reactions could be seen. For example, strains Cornell 56 and 57 of Lancefield group G were easily distinguished by their DNA fingerprint pattern. Strains Cornell 14, 50, 81 and Kiel 10102 which are different by two bands in the DNA fingerprints gave precipitin

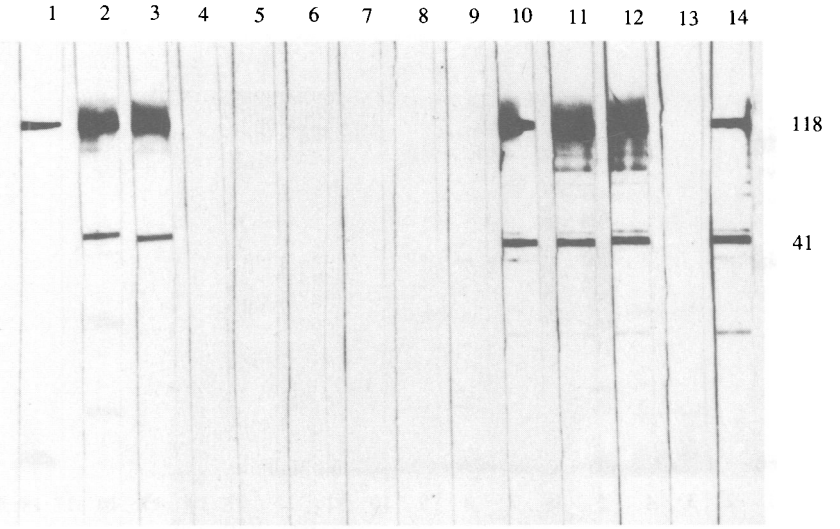

Fig. 7. Detection of SDS-heat extracted antigens of *S. uberis* strain ATCC 19436 by antiserum to *S. uberis* strain R2 previously absorbed three times with cells of *S. pyogenes A* type M12 (1), *S. agalactiae* strains DL and 64-149 (2, 3), *S. dysgalactiae* strains GM2 and SC1 (4, 5), *S. uberis* strain ATCC 19436 (6), *S. zooepidemicus* strain W60 (7), *S. equisimilis* strain Hipkin (8), *S. canis* DAUT1 (9), *S. bovis* WINT1 (10), *E. faecalis* WINT2 (11) and *S. lactis* ssp. *lactis* WINT3 (12). The immunoblots were developed in peroxidase conjugated goat anti-rabbit serum and protein G followed by 4-chloro-1-naphthol. Strip 14 was incubated with untreated R2 serum and strip 13 was a conjugate control for non-specific IgG binding. Numbers on the left locate the M_r of major antigens.

reactions in either group B, E, P or were non-groupable. Furthermore, no correlation between DNA fingerprints and immunoblot fingerprints was evident. DNA fingerprints of *S. parauberis* NCDO 2020 were unique and did not match those of *S. uberis* (Fig. 8*b*).

DISCUSSION

The species *S. uberis*, though clinically very important, is bacteriologically only vaguely defined. The immunoblots and DNA fingerprints in this study provide strong evidence for conservation and uniformity among *S. uberis* strains isolated from the bovine udder and show that they are markedly different from the type strain of *S. parauberis*. We therefore support the introduction of *S. uberis* subtype II as *S. parauberis* sp. nov. as recently proposed following comparative analysis of 16S ribosomal RNA sequences [13]. Both species have identical biochemical properties, i.e. hydrolysis of sodium hippurate and production of acid from cellobiose, esculin, glucose, fructose, galactose, inulin, maltose, mannitol, mannose, ribose, salicin, sorbitol, starch, sucrose and trehalose, but not from arabinose erythritol, glycerol, sorbose, and xylose. Variations occur in the ability of *S. parauberis* strains to utilize sodium hippurate, inulin, mannitol, sorbitol, ribose, and raffinose. The ability of *S. parauberis* to grow at 10 °C is the only consistent biochemical or cultural characteristic by which it can be distinguished from *S. uberis*.

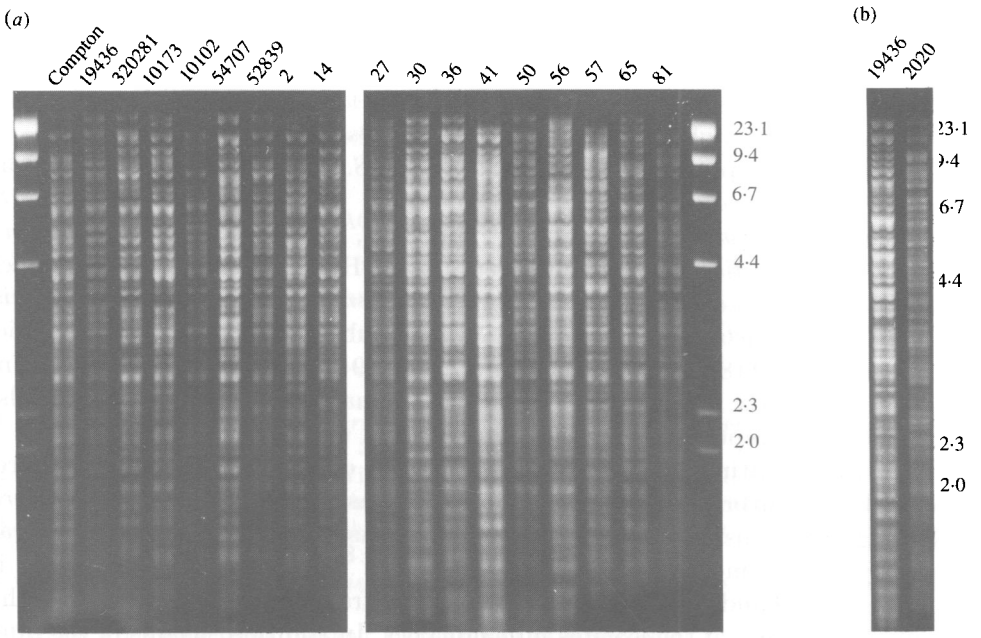

Fig. 8. (*a*) DNA fingerprints (*Hin*d III) of *S. uberis* strains Compton and ATCC 19436, strains Kiel 320281, 10173, 10102, 54707 and 52839 and Cornell 2, 14, 27, 30, 36, 41, 50, 56, 57, 65 and 81. Portions of two gels are shown. The numbers on the right are the M_r in kilobase pairs of bacteriophage λ-DNA fragments after *Hin*d III digestion. (*b*) DNA fingerprints (*Hin*dIII) of *S. uberis*-type strain ATCC 19436 and *S. parauberis* sp. nov. strain NCDO 2020.

The *S. uberis* strains exhibited almost identical immunoblot fingerprints of the major antigens of approximately 40–41, 59–61 and 118–122 kDa following reaction with rabbit antisera. Antibodies to the 40–41 and 118–122 kDa antigens occurred both in sera of dairy cows with no current clinical evidence of mastitis and in milk of cows previously infected with *S. uberis*. Ten bovine sera gave positive immunoblotting reactions that were presumably derived from field exposure to strains of *S. uberis* of the same serological diversity as the strains investigated. They reacted consistently with the same antigens in 18 different *S. uberis* strains of Lancefield groups B, E, G or P or that were non-groupable. Immunoblot fingerprint patterns of *S. uberis* were different from those of *S. parauberis* and antiserum to the *S. parauberis*-type strain NCDO 2020 reacted only weakly with *S. uberis* ATCC 19436. Immunoblot fingerprinting was introduced by Poxton and co-workers [26] as a powerful epidemiological tool for discriminating between bacterial strains of the same species. The technique detects differences in banding patterns of crude extracts immunoblotted with polyvalent antisera and has been successfully employed to cluster *Aspergillus fumigatus* [27], coagulase-negativer and methicillin-resistant staphylococci [28], *Staphylococcus aureus* [29, 30], *Campylobacter pylori* [31], *Candida albicans* [32] and *Clostridium difficile* [33, 34]. Immunoblot fingerprints provide easily interpreted patterns for isolates that are virtually indistinguishable by Coomassie blue or silver stained SDS–PAGE gels. The consistent immunoblot fingerprinting pattern of *S. uberis*

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appears to be unique and therefore should be useful in defining the species. The minor variations of up to 2 kDa in the M_r of some antigens were apparently randomly distributed and so could not be used in defining subtypes. Similar size variations in protein antigens resulting from deletion and addition of repeat sequences have been observed in groups A and C streptococci [35].

The immunoblot patterns of both *S. uberis* and *S. parauberis* were distinct from those of *S. pyogenes*-type M12, *S. agalactiae*, *S. dysgalactiae*, *S. equi*, *S. zooepidemicus*, *S. equisimilis*, group G streptococci, *E. faecalis*, *S. bovis*, *S. durans*, *S. lactis* subsp. *lactis* and viridans streptococci. However, antigens on the cell surface of *S. pyogenes*, *S. dysgalactiae*, *S. zooepidemicus*, *S. equisimilis*, *S. canis*, but not of *S. agalactiae*, *E. faecalis* and *S. lactis* subsp. *lactis* absorbed antibodies to the 40, 61 and 118 kDa bands of strain ATCC 19436 from rabbit hyperimmune serum to *S. uberis*. This suggests that determinants on these bands are also distributed on streptococcal species other than *S. uberis*.

DNA fingerprinting has been suggested as an epidemiological tool in source tracing during outbreaks of *S. uberis* mediated mastitis [36]. Analysis of *S. uberis* DNA fingerprints using the restriction endonuclease *Hin*d III revealed an overall similarity of fragment profile (Fig. 7). Strains showed a few differences in polynucleotide bands with at least two unique fragments for each strain. This precluded grouping of the isolates into subtypes. In addition, strains of the same Lancefield group had slightly different fragment profiles. The fingerprints of *S. parauberis* and *S. uberis* were substantially different, although showing more similarity than reported to exist between *S. uberis*, *S. bovis* and *S. fecalis* [36]. DNA fingerprinting is effective in distinguishing closely related Gram-positive cocci and has disclosed major heterogeneity within subtypes of *S. pneumoniae* and *S. pyogenes* [37, 38], and among *S. zooepidemicus* [39], coagulase-negative staphylococci [28, 40] and *S. mutans* [41].

Lancefield grouping was of little value in defining *S. uberis*. Various grouping reactions were seen that were unrelated to the immunoblot and DNA fingerprints. *S. uberis* strains isolated in the United States carried Lancefield antigens of groups E and P. The percentage of group E-positive strains (14·3%) was similar to that found in former investigations [14], while the number of strains reacting to group P antiserum (20%) was surprisingly high. In addition, American strains reacting with Lancefield groups B (2) and/or G antisera (3) were recorded for the first time. *S. parauberis* NCDO 2020 showed a weak reaction with group G antiserum.

Little is known about the cell-wall polysaccharide antigens responsible for the Lancefield grouping reaction of *S uberis*. Rhamnose seems to be an important component of the immunodeterminants. Group E carbohydrate antigen consists of a poly-L-rhamnose backbone of alternating O-2- and O-3-linked rhamnose units with immunodominent terminal β-D-glucopyranosyl groups. Rhamnose also contributes to the immunodominant structure of group B and G streptococci which may explain the occasional serological cross-reactions that occur between these groups [42]. The diversity of Lancefield antigens found in *S uberis* may reflect only very slight differences in configuration of the carbohydrate epitopes.

Thus, *S. uberis* is much more homogeneous in immunoblot and DNA fingerprints and Lancefield grouping would suggest and can easily be distinguished by these techniques from *S. parauberis*. Further studies should focus on immunoblot and

DNA fingerprints of a larger number of *S. parauberis* strains to provide more information about genetic and antigenic variations in this new species. Further development of these tools for rapid discrimination of *S. uberis* and *S. parauberis* strains will be helpful in the study of the epidemiologic features of mastitis caused by each species and in rapid identification methods.

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