Isolation and Characterization of a Second Isolate of Streptococcus iniae

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A second strain of Streptococcus iniae has been recovered from an Amazon freshwater dolphin (Inia geoffrensis). This isolate differs from the first-described isolate in its ability to produce acid from lactose but not salicin and its inability to hydrolyze esculin. The two isolates share a common cell wall antigen that appears to represent the C polysaccharide grouping antigen of this species. In addition, there are strain-specific antigens associated with each isolate. The second strain has been designated strain BU (= ATCC 29177).

We previously (3) characterized a single strain of a new isolate of beta-hemolytic streptococcus obtained from dermal lesions on an Amazon freshwater dolphin, Inia geoffrensis, housed in the Steinhardt Aquarium in San Francisco, Calif. Subsequently, a second isolate of beta-hemolytic streptococcus was obtained from a swab of the interior wall of a dermal lesion on another specimen of I. geoffrensis housed at the Niagara Falls Aquarium in New York. This second strain, designated BU, was found to have numerous similarities to the first isolate, the most important being a serologically identical cell wall antigen that appears to represent the C polysaccharide of this species. Differences were noted, however, in some sugar fermentation patterns and biochemical reactions as well as in antigens. The characters of the second isolate of Streptococcus iniae are presented as further evidence that these two organisms represent a new species and serogroup of Streptococcus.

MATERIALS AND METHODS

Bacterial strains. Strain PW, the previously described type strain of S. iniae (3), was compared with the second isolate of this species, strain BU.

Methods. Maintenance and growth of cultures, morphological and physiological studies, antibiotic susceptibility testing, determination of deoxyribonucleic acid (DNA) base composition, preparation of antisera and seroassays, and animal studies were all performed as previously described (3). The three additional extraction procedures were: (i) 2% trypsin in 0.05 M tri(hydroxymethyl)aminomethane (Tris) with 0.0115 M calcium chloride (pH 8); (ii) 2% pepsin in 0.1 M sodium acetate (pH 4); and (iii) 10% trichloroacetic acid. The proteolytic enzyme extractions were performed at 37°C for 24 h, and the trichloroacetic acid extraction was performed at 4°C for 24 h. Cells were removed by centrifugation and the supernatants were brought to pH 7.4.

Chemical analysis. The protein contents of the antigen extracts were measured by the method of Lowry et al., using bovine serum albumin as the standard (2), and the carbohydrate contents were estimated by the phenol-sulfuric acid method, using dextran 50 as the standard (1).

Serology. Rabbit antisera to each S. iniae strain, prepared as previously described (3), were adsorbed twice for 1 h at 37°C and overnight at 4°C with a washed and pelleted overnight culture of the heterologous S. iniae strain grown in 40 ml of Todd-Hewitt broth at 37°C.

Serum obtained from the Niagara Falls dolphin was tested for the presence of antibodies to S. iniae antigens by double diffusion in agar.

RESULTS

Morphology. Cultures of strain BU formed chains of gram-positive cocci and produced indented, or "bulls-eye," colonies on sheep blood agar. In Todd-Hewitt broth, coarse, granular growth was confined mostly to the bottom of the tube. These growth characteristics are identical to those described for strain PW.

Biochemical and fermentation characteristics. Strain BU produced acid from dextran,
fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sucrose, and trehalose. No change of pH occurred when the organism was grown in the presence of arabinose, dulcitol, glycerol, inositol, inulin, melibiose, raffinose, rhamnose, salicin, sorbitol, or xylose. Strain PW differs from strain BU in its ability to produce acid from salicin but not from lactose. Table 1 presents the biochemical reactions of the two strains in selected test media. Except for the hydrolysis of esculin, all reactions are the same for the two strains.

Antibiotic susceptibility. The patterns of the antibiotic susceptibilities of the two strains were identical, including susceptibility to gentamicin and resistance to kanamycin, neomycin, and streptomycin (3).

DNA base composition. The melting temperature of the DNA isolated from strain BU was 82.9°C, which corresponds to a guanine-plus-cytosine content of 32.9 mol%, which is identical to the value calculated for strain PW.

Animal virulence studies. Rabbits, mice, and guinea pigs were all resistant to intravenous, subcutaneous, and intraperitoneal injections with up to $10^8$ colony-forming units of strain BU.

Hemolysis production. When grown on sheep blood agar, both strains produced a small zone of beta-hemolysis surrounded by a diffuse outer zone of alpha reaction. Similar patterns of hemolysis were observed when the organisms were grown on human, rabbit, or guinea pig blood agar. However, when grown on freshwater dolphin blood, the area of beta-hemolysis was two to five times greater than that produced on blood from other species (Fig. 1), and additionally no zone of alpha reaction was seen. Whether this represents a greater affinity of S. iniae hemolysins for freshwater-dolphin erythrocytes or a greater susceptibility of these cells to lysis in general is not known.

Serological studies. In double-diffusion gels, rabbit antiserum to strain PW demonstrated the presence of two antigens in HCl, trypsin, pepsin, lytic enzyme, and autoclave extracts of strain PW but only a single antigen in trichloroacetic acid and formamide extracts (see Table 2). This single antigen from the trichloroacetic acid and formamide extracts gave a reaction of identity with one of the two antigens present in the other extracts. After absorption of the antiserum with the heterologous strain BU, the sera reacted only with the second antigen present in the HCl, trypsin, pepsin, lytic enzyme, and autoclave extracts of strain PW and not with the common

![Fig. 1](image-url)
antigen present in all seven extracts (Table 2).

Antisera to strain BU, when tested against BU-antigen extracts in double-diffusion gels, also showed the presence of two antigens in HCl, lytic enzyme, and autoclave extracts but only a single antigen in trichloroacetic acid, formamide, trypsin, and pepsin extracts. The single antigen in the trichloroacetic acid and formamide extracts again gave a reaction of identity with one of the two antigens present in the HCl, lytic enzyme, and autoclave extracts but not with the single antigen present in the trypsin and pepsin extracts. These latter antigens, moreover, appeared identical with the second antigen present in HCl, lytic enzyme, and autoclave extracts. Absorption of antisera to strain BU with strain PW cells removed only the reactivity to the common antigen present in the trichloroacetic acid and formamide extracts and not the reactivity to the second antigen found in the other five extracts (Table 2).

The presence of an antigen common to both strains was confirmed by reacting antisera to each strain with all seven extracts of the heterologous strain. Lines of identity were produced to the common antigen present in the formamide and trichloroacetic acid extracts of both strains but not to the second antigen which was absent in these two extracts. Furthermore, antisera to either strain reacted identically to the formamide and trichloroacetic acid extracts of both strains when tested together (Table 2). Thus, there appears to be a common grouping antigen present in all seven extracts of strain PW and in all but the trypsin and pepsin extracts of strain BU, whereas strain-specific antigens appear in all but the trichloroacetic acid and formamide extracts of both strains.

Gel diffusion also revealed that serum from the *I. geoffrensis* specimen in Niagara Falls reacted with the trypsin and autoclave extracts of strain BU with which it was infected and weakly with the trypsin extracts of strain PW (Fig. 2).

**Fig. 2. Reaction of dolphin serum with antigen extracts of *S. iniae*. In center well is dolphin serum. Left-hand pattern, Extracts from strain BU; right-hand pattern, extracts from strain PW: (a) HCl extract; (b) trypsin extract; (c) trichloroacetic acid extract; (d) autoclave extract; (e) pepsin extract; (f) lytic enzyme extract.**

**Chemical analysis.** Table 3 shows the protein and carbohydrate contents of the seven antigen preparations extracted from 100 mg of freeze-dried cells. As expected, there is no detectable protein in the trichloroacetic acid and formamide extracts, suggesting that the common grouping antigen present in these extracts is most likely a polysaccharide, whereas the presence of protein in the other antigen extracts suggests that the strain-specific antigens may be a protein. Finally, the trypsin and pepsin extracts of strain BU, which appeared to lack serologically detectable grouping antigen, contained no detectable carbohydrate.

**Corroboration of new serogroup status.** To assess the biological and serological properties of *S. iniae* BU and to compare them to those of established species of streptococci, strain BU was submitted to R. R. Facklam and R. L. Wood. Both laboratories confirmed the properties of the strain reported here as well as the serological identity of the grouping antigen of the two *S. iniae* strains and its uniqueness from the grouping antigens of the established serogroups of streptococci.
The isolation and description of a second S. iniae strain with morphological properties, DNA base composition, and a grouping antigen identical to those of the first isolate should add strength to the acceptance of S. iniae as a new species and serogroup of Streptococcus. Strain BU has been deposited in the American Type Culture Collection (ATCC), Rockville, Md., under the accession number 29177.

The biochemical and serological differences between the two strains of S. iniae necessitate a modification of the description of this species. Variable fermentation of lactose and salicin, as well as variable hydrolysis of esculin, now characterizes S. iniae, whereas the lack of fermentation of sorbitol distinguishes it from Streptococcus infrequens groups E, P, and U.

The isolation of both strains of S. iniae from dermal lesions on specimens of I. geoffrensis is further evidence that these organisms represent a new species and serogroup and strengthens its association with the dermatosis in I. geoffrensis known as "golf ball disease." The strong hemolysis shown by growth of S. iniae on I. geoffrensis blood as well as the finding of antibodies in the sera from one of the animals tends to support a pathogenic role for S. iniae in Amazon freshwater dolphins.

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