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Observation of Beta-Hemolysis Among Three Strains of *Streptococcus mutans*

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Streptococcus mutans is normally alpha- or gamma-hemolytic on blood agar plates. However, three recently isolated *S. mutans* strains were observed to elicit beta-hemolysis. The production and nature of a hemolytic substance were studied.

The occurrence of beta-hemolytic streptococci in the oral cavity is of considerable interest because of their possible role in the spread of infection (30). Group A beta-hemolytic streptococci are the cause of such diseases as pharyngitis, tonsilitis, scarlet fever, and rheumatic fever in human beings. Certain extracellular products elaborated by group A streptococci (including the hemolysins, streptolysin O, and streptolysin S [35]) have been related to the pathogenesis of these diseases.

Although certain groups of streptococci (A, C, and G) are well known for their hemolysin production (4), reports of beta-hemolysis among *Streptococcus mutans* are scarce. *S. mutans* is characteristically alpha- or gamma-hemolytic on blood agar (14). However, Perch et al. (32) observed that 16 of 54 strains, isolated from blood of patients with subacute bacterial endocarditis or from human teeth, exhibited varying degrees of beta-hemolysis on blood agar plates. Facklam (13), while studying characteristics of *S. mutans* from human dental plaque and blood, found 16 of 142 *S. mutans* strains exhibiting beta-hemolysis. However, other than these isolated reports, little is known about the beta-hemolytic phenomenon among *S. mutans*.

Of 47 *S. mutans* strains initially isolated from human dental plaque samples plated on mitis salivarius agar (Difco Laboratories) and subsequently cloned on blood agar plates, three were found to exhibit beta-hemolysis. The beta-hemolysis observed was not visibly different than that seen with the group A streptococci. These three strains were classified as biotype b (strains 415 and 841) or d (strain 339) by the biochemical scheme of Shklair and Keene (33). These biotypes correspond to the *b* and *d* serotypes, respectively, of Bratthall (5) and the genetic groups II and III of Coykendall (8), respectively.

The medium used for culture growth and maintenance, as well as for hemolysin production, was Todd-Hewitt (Difco). For preparation of plates and slants, agar was added to a final concentration of 1.5%. Cells from an early-stationary-phase culture of beta-hemolytic *S. mutans* in Todd-Hewitt broth were harvested by centrifugation; the remaining supernatant was assayed for hemolytic activity and stored at −20°C. *S. mutans* 841 produced significantly more hemolysin than strain 415 or 339 and was used exclusively in these experiments unless noted otherwise.

Hemolysin was produced optimally in Todd-Hewitt broth culture, with less hemolytic activity observed when cells were grown in Trypticase soy broth (Baltimore Biological Laboratory). No hemolytic activity was obtained in supernatants of cells grown in brain heart infusion broth, Trypticase soy broth (with phosphate buffer; final concentration, 0.1 M), or thioglycolate broth (Difco). However, when these broth media were supplemented with 1.5% agar and 5% sheep blood, beta-hemolysis was clearly observable on all blood agar plates. In addition, beta-hemolysis from strains 415 and 841 was detected in Todd-Hewitt agar supplemented with either 5% sheep, human, or rabbit blood.

For the assay of *S. mutans* hemolysin, a procedure similar to that used by Duncan and Mason (9) to assay streptolysin S was utilized. Difibrinated sheep blood (Texas Biological) was centrifuged, the plasma was aspirated, and the erythrocytes were washed three times in phosphate-buffered saline (NaCl, 0.126 M; KH_2PO_4, 0.023 M; Na_2HPO_4, 0.013 M; pH 6.5) and suspended in phosphate-buffered saline to a final concentration of 0.6%. One hemolytic unit is defined as the amount of *S. mutans* hemolysin that produces complete lysis of a 0.6% sheep erythrocyte suspension after 30 min at 37°C. A 1.0% saponin solution (0.05 ml) was added to the same volume of an erythrocyte suspension for the preparation of a complete hemolysis standard. Lysis of the erythrocyte suspension was monitored colorimetrically at 540 nm with a

Spectronic 20 spectrophotometer.

Hemolysin production was first detected in early-stationary-phase cultures, with optimal hemolytic activity observed when the pH of the culture dropped to 5.5 (Fig. 1). When the cells advanced well into the stationary phase and the pH dropped below 5.0, the hemolytic activity decreased (data not shown). This finding suggests that the hemolysin is unstable at a low pH, and, with increased acid accumulation in the stationary-phase culture, decreased hemolysin activity is observed. In fact, when the cell-free hemolytic factor was exposed to a pH below 5.5 for 20 min at 37°C and readjusted to pH 5.5, activity was lost. Similarly, raising the pH above 5.5 and readjusting it to 5.5 resulted in loss of activity. A complete loss of hemolysin activity was not observed unless the pH was less than 3.0 or greater than 10.0.

Todd-Hewitt broth adjusted to pH 5.0 did not lyse erythrocytes in our assay. The hemolytic substance in phosphate-buffered saline lysed erythrocytes. These findings indicate that the beta-hemolysis caused by *S. mutans* was not due to an alteration in pH.

Trypsin (type III from bovine pancreas), hyaluronidase, cholesterol, and albumin (Sigma); potassium Pronase, catalase ribonuclease, and deoxyribonuclease (Calbiochem); and dithiothreitol (J. T. Baker, Phillipsburg, N.J.) were the enzymes and chemicals tested for their effect on *S. mutans* hemolysin. They were dissolved in phosphate-buffered saline and mixed with 2 hemolytic units of hemolysin, with a 100-μg/ml final concentration of all enzymes and chemicals. All tubes were incubated for 30 min at 37°C and then assayed for activity. No loss of hemolytic activity was observed as a result of incubation with these compounds.

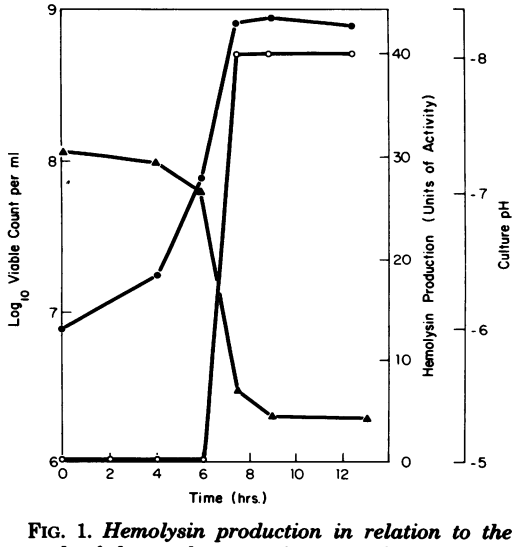

FIG. 1. *Hemolysin production in relation to the growth of the producer strain 841 under anaerobic conditions in Todd-Hewitt broth. Symbols:* (●) *viable count;* (○) *hemolysin production;* (▲) *culture pH.*

Heating at 100°C for 1 h did not cause loss of activity, and the hemolysin appeared stable for up to 4 months at −20°C. Furthermore, the hemolytic substance was dialyzable, suggesting that the hemolysin may have a molecular weight of less than 10,000.

Hydrogen peroxide produced under aerobic conditions (35) by many microorganisms, including streptococci (17), has been observed to cause hemolysis (34). Because the *S. mutans* strains were incubated under strict anaerobic conditions for the production of the hemolysin and the hemolysin was resistant to catalase, the hemolysin was not hydrogen peroxide.

When the hemolysin was incubated with cholesterol and subsequently incubated with sheep erythrocytes, no decrease in *S. mutans* hemolytic activity was observed. This observation, together with the protease resistance of the hemolysin and its low molecular weight, suggests that the *S. mutans* hemolysin is unlike the streptococcal group A streptolysin O hemolysin studied by Oberley and Duncan (31). On the other hand, the lower molecular weight and oxygen stability of the *S. mutans* hemolysin may denote a molecule similar to the group A streptolysin S hemolysin (4).

The virulence of *S. mutans* in relation to the occurrence of dental caries in humans has been well documented (6, 12, 21, 23, 27). It has also been reported that streptococci harbored in the oral cavities of humans are etiological agents of bacterial endocarditis and subacute bacterial endocarditis (18, 19, 24). In fact, the pathogenic role *S. mutans* may play in bacteremia or bacteremia leading to bacterial endocarditis has been reported by several authors (1, 2, 15, 22, 29; C. E. DeMoor, J. D. DeStoppelaar, and J. van Houte, Caries Res. **6**:73, 1973).

The biological role of the *S. mutans* hemolysin is unknown. On the other hand, it is well known that the hemolysins, streptolysins O and S, elaborated by group A streptococci have been related to the pathogenesis of disease (35). Whether these substances serve as agents to increase the virulence of *S. mutans,* act as by-products in normal cellular growth, or serve in some other capacity remains to be answered.

The occurrence of bacterial plasmids among streptococci has been reported. They have been shown to code for antibiotic resistance (7), bacteriocin production (20), lactose fermentation

(28), and proteinase production (25). Recent reports from various laboratories have demonstrated plasmid deoxyribonucleic acid in several strains of *S. mutans* (10, 26). Although no direct evidence of phenotypic characters coded for plasmids was reported, Higuchi et al. (16) have suggested that synthesis of insoluble extracellular polysaccharides by *S. mutans* may be attributed to plasmids. Furthermore, hemolysin production by *S. faecalis* has been shown to be coded by a plasmid (11). If the *S. mutans* hemolysin were similarly shown to be coded by a plasmid, this could serve as a valuable tool in determining phenotypic properties coded by the *S. mutans* plasmid.

The source of *S. mutans* in human oral flora has not been definitely established. However, in a recent study using bacteriocin typing, a case for maternal transfer of *S. mutans* was established (3). With the *S. mutans* beta-hemolysin as a marker, the route by which cariogenic bacteria are established in the human population may be studied. "Hemolysin typing" may serve as a useful epidemiological tool in studies concerned with human transmission of *S. mutans* or in tracing the infectious process of dental caries from one area of the oral cavity to another in the same individual.

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