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Biochemical Properties of CO_2-Dependent Streptococci

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A total of 153 clinical isolates and 10 reference strains were employed in an investigation of CO_2-dependent streptococci. Their selection was based on a lactic acid homofermentative end product. This group of organisms grew best in 5 to 10% CO_2, and several species, including *Streptococcus mutans*, *S. intermedius*, *Streptococcus* MG, *S. anginosus*, and *S. constellatus*, required increased CO_2 for primary recovery. A basal medium of thioglycolate with 0.1% Tween 80 and phenol red was prepared and used with selected carbohydrates. This media provided luxuriant growth. Serological testing failed to give any definitive correlation with species identification. A shortened differentiation scheme, combined from previous studies, was proposed.

Many streptococci which require CO_2 for growth are isolated in clinical bacteriology laboratories. These bacteria have been given many synonyms, e.g., microaerophilic, capnophilic, aerotolerant, and CO_2 dependent. We have elected to study CO_2-dependent streptococci as a group and to describe streptococci which grow best on blood agar medium incubated in 5 to 10% CO_2.

CO_2-dependent streptococci are a heterogeneous group which include beta-hemolytic streptococci, often called minute beta or indifferent streptococci (14, 16); viridans and nonhemolytic streptococci; and some previously designated anaerobic cocci (8, 9, 20). It has been observed that some streptococci, e.g., *Streptococcus mutans*, may appear as obligate anaerobes upon primary isolation but later become facultative. Also, some streptococci rapidly lose their dependency for CO_2 after subculturing. Our study will describe the characteristics of streptococci which are fastidious and do not readily lose their requirement for CO_2.

MATERIALS AND METHODS

Strains. The clinical isolates were obtained from the following institutions: Long Beach Administration Hospital, Long Beach, Calif.; Wadsworth Anaerobic Bacteriology Laboratory, Los Angeles, Calif.; Cedar-Sinai Medical Center, Los Angeles, Calif.; the University of California at Los Angeles, Los Angeles, Calif.; and San Francisco General Hospital (SFGH), San Francisco, Calif. (Table 1). The reference strains were acquired from the American Type Culture Collection (ATCC) and various reference laboratories as follows: *Streptococcus anginosus*, Wadsworth VA; *Streptococcus intermedius* ATCC 27335; *Streptococcus constellatus* ATCC 27513; *Streptococcus morbillorum*, SFGH stock; *Streptococcus mitis* ATCC 6429 and ATCC 15914; *Streptococcus salivarius* ATCC 13419; *Streptococcus sanguis* I, ATCC 10556; *S. sanguis* II, ATCC 10557; *Streptococcus mutans*, Center for Disease Control. In total, 153 clinical isolates and 10 reference strains were studied.

Stock cultures. Working cultures were maintained in cooked-meat medium (BBL Microbiology Systems) and held at room temperature. Permanent stocks were either lyophilized in sterile skim milk (Difco) or frozen in Brucella broth (BBL) with 15% glycerol and maintained at −70°C.

Hemolytic properties. Organisms were inoculated onto tryptic soy agar (Difco) with 5% sheep blood and incubated in a GasPak anaerobic system (BBL). Hemolysis was read after 48 h.

Gas-liquid chromatography. All organisms were grown in peptone-yeast-glucose broth for gas-liquid chromatographic analysis. After 3 days of incubation, or when adequate growth was obtained, culture extracts for short-chain fatty acids and dicarboxylic acids were prepared and analyzed according to the Virginia Polytechnic Institute methodology (9). Those organisms producing lactate homofermentatively (with or without small amounts of acetic acid) were considered streptococci and were included in the study.

Composition of media. A basal medium was prepared containing fluid thioglycolate (without indicator or glucose; BBL), 0.1% Tween 80, and a 0.04% solution of phenol red indicator. Carbohydrates were added to the basal medium to give a 1.0% concentration, and the pH was adjusted to 7.2. The carbohydrate broths were dispensed into screw-capped tubes (13 by 100 mm) and autoclaved for 12 min at 121°C. After sterilization, the tubes were stored at room temperature in the dark and used within 7 days. Esculin hydrolysis was determined in the basal medium containing 0.03% esculin without a phenol red indicator. A 1% ferric ammonium citrate solution was used to detect esculin hydrolysis. Biochemical media were inoculated from an overnight growth of the organism in fluid thioglycolate broth (BBL) containing 0.1% Tween 80. Test reactions were interpreted after 72 h of incubation without added CO_2. The preparation and interpretation of dextran production on 5% sucrose agar and

deoxyribonuclease production have been previously described (3). The plates were incubated in an incubator with 5 to 10% CO_2.

Serotyping. Isolates were grown in 1% glucose-supplemented Todd-Hewitt broth (BBL) for the preparation of Lancefield extracts for serogrouping by the Lancefield method (11, 12). The Rantz and Randall autoclave method for the extraction of the group antigen, and precipitin grouping using the capillary tube method, were used (21). Antisera for streptococcal groups A, B, C, D, E, F, G, H, K, and MG were purchased from Difco Laboratories.

RESULTS

All organisms tested grew well aerobically in an incubator with 5 to 10% CO_2. The results of the biochemical tests of the reference strains and clinical isolates are shown in Table 2. All strains tested produced acid from glucose. *S. mutans* was characterized by its ability to produce acid in inulin, lactose, mannitol, raffinose, sorbitol, maltose, and sucrose. All strains tested hydrolyzed esculin and demonstrated dextran production of 5% sucrose agar. None of the seven strains tested was beta-hemolytic.

S. intermedius, Streptococcus MG, and *S. anginosus* are listed as one group since they demonstrated common biochemical reactions. These isolates were most often nonhemolytic; however, 9 of the 51 isolates were alpha-hemolytic, and 7 strains were beta-hemolytic. The distinguishing characteristics included the ability to form acid in lactose, maltose, sucrose, and (rarely) in raffinose, and inability to form acid in inulin, mannitol, and sorbitol. Ninety-eight percent of the isolates hydrolyzed esculin. Twenty-one isolates produced extracellular deoxyribonuclease.

S. constellatus was distinguished from the *S. intermedius-Streptococcus* MG-*S. anginosus* group by its failure to produce acid from lactose and inability to curd litmus milk. *S. morbillorum* was differentiated from *S. constellatus* by its failure to grow in litmus milk and failure to hydrolyze esculin.

The other CO_2-dependent streptococci include *S. mitis, S. salivarius, S. sanguis* I, and *S. sanguis* II. They were differentiated on the basis of four reactions: ability to produce acid from inulin and raffinose fermentation, esculin hydrolysis, and dextran production on 5% sucrose agar.

TABLE 1. *Clinical sources of CO_2-dependent streptococci*

Source	Total no.
Abscess	33
Blood	96
Bone marrow	1
Cerebrospinal fluid	11
Gall bladder	5
Pleural fluid	5
Lung	2

TABLE 2. *Percentage of CO_2-dependent streptococci giving positive reactions*

Test	*S. mutans* (7)[a]	*S. intermedius–Streptococcus* MG–*S. anginosus* group (51)	*S. constellatus* (18)	*S. morbillorum* (3)	*S. mitis* (29)	*S. salivarius* (4)	*S. sanguis* I (22)	*S. sanguis* II (30)
Hemolysis								
Alpha	14	18	17	0	59	0	68	75
Beta	0	14	50	0	0	0	0	0
Gamma	86	68	33	100	41	100	32	25
Glucose	100	100	100	100	100	100	100	100
Inulin	86	0	0	0	0	100	100	0
Lactose	100	100	0	0	100	100	100	100
Maltose	100	100	100	100	100	100	100	100
Mannitol	100	0	0	0	0	0	0	0
Raffinose	71	20	0	0	0	100	36	100
Sorbitol	71	0	0	0	0	0	0	0
Sucrose	100	100	100	100	100	100	100	100
Esculin hydrolysis	100	98	61	0	0	100	86	0
Litmus milk								
Reduction	100	100	100	0	100	100	100	100
Curd	100	100	0	100	100	100	100	100
Deoxyribonuclease	0	41	11	0	0	0	0	0
5% Sucrose agar								
Adherent	100	4	0	0	3	0	77	75
Nonadherent	0	96	100	100	97	100	23	25

[a] Number of strains tested.

The results of the Lancefield groupings are shown in Table 3. Not all isolates studied were typable. None of the 163 organisms tested reacted with group A, B, or D. *S. mutans, S. morbillorum,* and *S. salivarius* did not react with any grouping antisera. *S. intermedius* ATCC 27335 did not react with any antisera tested. However, stock strain *S. anginosus* demonstrated the group F antigen. Nine of the 51 clinical strains of the *S. intermedius-Streptococcus* MG-*S. anginosus* group reacted with group F antiserum. Four of these isolates were beta-hemolytic and five were nonhemolytic on sheep blood agar. In addition, three isolates of the *S. intermedius-Streptococcus* MG-*S. anginosus* group reacted with group MG, and six showed cross-reactions with group F and group MG.

The *S. constellatus* ATCC 27513 extract did not group with any antisera tested. Extracts from clinical isolates gave varied groupings. Of the nine beta-hemolytic strains of *S. constellatus* tested, one reacted with group E and one reacted with group K. Eleven strains were nonreactive with all antisera tested.

Extracts from *S. mitis* strains ATCC 6249 and ATCC 15914 reacted with group G. However, clinical isolates of *S. mitis* did not demonstrate any consistent grouping. *S. sanguis* I, ATCC 10556, reacted with group H, as did four clinical isolates tested. One clinical isolate of *S. sanguis* I reacted with group MG. The reference extract of *S. sanguis* II, ATCC 10557, did not react with any groups tested. Of the 28 clinical isolates of *S. sanguis* II tested, 27 were nonreactive with the groups tested, and one reacted with group K.

DISCUSSION

This study was performed to categorize streptococci which are frequently called microaerophilic or CO_2 dependent and to suggest a different basal medium for physiological characterization. The thioglycolate medium offers the organisms a reduced oxygen environment which enhances growth. Shklair and Keene used a thioglycolate basal medium with a purple broth base (23) in their studies with *S. mutans.* The addition of 0.1% Tween 80 to thioglycolate also enhanced the growth of streptococci, and the use of phenol red as an indicator proved to be more sensitive for small amounts of acid production than bromocresol purple.

Facklam has made much progress in the classification of viridans streptococci (4). In his study, the viridans streptococci are defined as streptococci which are alpha or nonhemolytic on blood agar and are susceptible to penicillin (4). There are CO_2-dependent strains within species of viridans streptococci, which include *S. salivarius, S. mitis, S. mutans, S. sanguis, S. intermedius, S. anginosus,* and *Streptococcus* MG. Whereas the original interest in this group was based on their involvement in infected dental root canals and their role in the pathogenesis of dental caries (7, 16), they are common causes of many infections (4, 23).

The splitting of *S. sanguis* into biotypes I and II has been suggested by Facklam (4). The major difference is the acid production in inulin by *S. sanguis* I and the acid production in raffinose by *S. sanguis* II (4). A high percentage of both biotypes produce dextrans on 5% sucrose agar.

S. anginosus, also called minute beta-hemolytic and included by Facklam in the viridans streptococci (4), is often characterized by its Lancefield group F or G reaction (13, 16). Ottens and Winkler (16) studied over 200 strains of nonhemolytic streptococci isolated from root canal cultures. It was found that 50% of the nonhemolytic strains belonged to Lancefield

TABLE 3. *Serological reactions of CO_2-dependent streptococci*

Lancefield group	*S. mutans*	*S. intermedius-Streptococcus* MG-*S. anginosus group*	*S. constellatus*	*S. morbillorum*	*S. mitis*	*S. salivarius*	*S. sanguis* I	*S. sanguis* II
A	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0
C	0	2	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0
E	0	0	1	0	0	0	0	0
F	0	9	3	0	0	0	0	0
G	0	0	2	0	2	0	0	1
H	0	0	0	0	0	1	5	0
K	0	0	1	0	0	0	0	1
MG	0	3	0	0	0	0	1	0
F/MG	0	6	0	0	0	0	0	0
Multiple	0	1	0	0	1	0	0	0
Nonreactive	7	30	11	3	26	3	16	27

group F, G, or C. In addition, it was shown by cross-absorption that the group antigen of the nonhemolytic group F strains was identical to the group antigen of the hemolytic strains of group F. A similar study by Poole and Wilson (17) examined 131 clinical isolates of minute-colony-forming beta-hemolytic streptococci of group F and G, also called *S. anginosus,* and the indifferent nonhemolytic streptococci of group F, G, or C, also called *Streptococcus* sp. MG. All showed similar cell wall composition and biochemical characteristics (17).

Streptococcus milleri was first isolated by Guthof (6) and further characterized by other investigators (2, 14, 15). It was considered a viridans streptococcus with characteristics similar to *Streptococcus* MG. Colman and Williams (2) considered *Streptococcus* MG the same species as *S. milleri.* Poole and Wilson (17) indicated that minute beta-hemolytic streptococci, or *S. anginosus,* are the same as *Streptococcus* MG and should be named *S. milleri.* Facklam (4) noted that the physiological characteristics of *S. intermedius* are similar to those of *Streptococcus* MG and suggested they be combined into one species. The conclusion reached was that *Streptococcus* MG, *S. intermedius, S. milleri* (K. C. Gross, M. P. Houghton, L. G. Senterfit, H. Masur, H. W. Murray, and R. B. Roberts, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C22, p. 39), and *S. anginosus* can all exhibit the same serological reactions and physiological characteristics. It is important to keep in mind that these organisms can be beta-hemolytic.

André Prévot in 1933 proposed an extensive classification of anaerobic bacteria. He described some of the anaerobic streptococci as strict anaerobes that upon subculture in vitro become facultative. *S. intermedius* and *Diplococcus constellatus* were considered strict anaerobes, and *S. morbillorum* was called a facultative anaerobe. This early scheme relied heavily on morphology and arrangement of cells (20). Hare et al. (7) arranged the anaerobic cocci into groups according to their metabolism and use of biochemicals. The CO_2-dependent streptococci were included in groups IV and IVa.

The anaerobic cocci were placed in the *Peptostreptococcus* and *Peptococcus* genera in 1936 (10). Differentiation was based primarily on morphology and cell arrangement. *Bergey's Manual of Determinative Bacteriology* (22) includes the CO_2-dependent streptococci under the genera *Peptostreptococcus* or *Peptococcus. S. intermedius* became *Peptostreptococcus intermedius,* and *D. constellatus* and *S. morbillorum* became *Peptococcus constellatus* and *Peptococcus morbillorum,* respectively. In 1974, Holdeman and Moore (8) suggested that *P. intermedius, P. constellatus,* and *P. morbillorum* be placed back in the genus *Streptococcus* on the basis of their production of lactate homofermentatively.

In conclusion, biochemical characterization appears to be a reliable method of identification for CO_2-dependent streptococci. It is also apparent that this group cannot be accurately identified by serological reactions. There is increasing understanding of the significance of the CO_2-dependent streptococci in human disease. Proper species identification is important for further understanding of the clinical significance of these streptococci. The system for identification presented is readily adapted to use in a clinical laboratory.

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