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Physiological Characterization of Nutritionally Variant Streptococci

ROBERT C. COOKSEY,[1]* FRANCES S. THOMPSON,[2] AND RICHARD R. FACKLAM[1]

Clinical Bacteriology Branch, Staphylococcus and Streptococcus Section,[1] and Enterobacteriology Branch, Anaerobe Section,[2] Bacteriology Division, Bureau of Laboratories, Center for Disease Control, Atlanta, Georgia 30333

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Twenty-five isolates of nutritionally variant streptococci submitted to the Streptococcus Laboratory of the Center for Disease Control over a 2-year period were tested for growth requirements and for biochemical reactions. After they were recovered from storage in blood at −170°C, all isolates grew within 48 h in both thioglycollate broth and Todd-Hewitt broth supplemented with 0.001% pyridoxal·HCl. They grew better in the latter, even though they all grew on unsupplemented infusion agar, anaerobe blood agar, and chopped meat-glucose medium. Biochemical patterns of the isolates resembled those of five viridans streptococcal species. Two isolates had patterns which did not resemble those of any viridans species. Biochemical reactions obtained with heart infusion broth base biochemicals and carbohydrate fermentation media compared favorably for an overall agreement rate of 86.5% for key tests. Lactic acid and acetic acid were the major fermentation products detected with gas-liquid chromatography.

The involvement of nutritionally variant streptococci in bacterial endocarditis and septicemia was reported in the early 1960's (7). Since then, descriptive names assigned to this group of organisms have included "satelliting streptococci," "thiol-dependent streptococci," "vitamin B_6-dependent streptococci," and "symbiotic streptococci." "Nutritionally variant streptococci" is an appropriate general term for this entire group since it does not indicate a specific metabolic deficiency.

Known growth requirements of the nutritionally variant streptococci indicate that thiol-containing compounds and various forms of vitamin B_6 are the required growth supplements. The organisms exhibit satellitism when seeded next to colonies of several gram-positive and gram-negative bacterial species. However, X and V factor disks do not enhance growth of the organisms, suggesting a deficiency in metabolites other than hemin or nicotinamide.

The few antimicrobial susceptibility studies of the nutritionally variant streptococci that have been reported suggest drug susceptibility patterns which resemble those of other viridans streptococci except for a somewhat greater susceptibility to streptomycin (1).

Because nutritionally variant streptococci are isolated so infrequently, it took 2 years to collect enough cultures to establish the biochemical characteristics of these streptococci.

MATERIALS AND METHODS

Strains. Of 28 streptococcal isolates submitted to the Streptococcus Reference Laboratory, Bacteriology Division of the Center for Disease Control (CDC), between June 1976 and June 1978, as either satelliting streptococci or thiol-dependent streptococci, 25 were successfully retrieved from storage. All had been isolated from the blood of patients. Of these 25 isolates, 19 were from patients with endocarditis, three from patients with sepsis, and three from patients with bacteremia. After they were received at CDC, the isolates were cultured in fluid thioglycollate medium (BBL Microbiology Systems) and on neopeptone infusion agar supplemented with 5% defibrinated rabbit blood onto which a single streak of *Staphylococcus epidermidis* was overlaid after seeding with the streptococcal isolate. After cultures were Gram stained, the organisms were identified by the system of Facklam (6). The isolates were stored in blood at −170°C. Subcultures were also sent to the Anaerobe Section, Bacteriology Division, for further testing.

Growth requirements. To provide optimal growth conditions, five liquid media were tested: unsupplemented Todd-Hewitt broth (THB), fluid thioglycollate medium (BBL Microbiology Systems), THB containing 5% defibrinated rabbit blood, Schaedler broth (BBL Microbiology Systems) supplemented with vitamin K_1 (0.1 μg/ml) and hemin (5.0 μg/ml), and THB supplemented with 0.001% pyridoxal·HCl. Pyridoxal·HCl, obtained from Sigma Chemical Co., St. Louis, Mo., was filter sterilized and stored at −20°C. Cultures were incubated at 37°C in candle extinction jars, and turbidity was visually judged after 24 and 48 h of incubation.

Biochemical testing. The physiological tests used in identifying the isolates are the standard biochemical procedures used in the Streptococcus Reference Laboratory for speciating alpha-hemolytic streptococci (6). Heart infusion broth base with added carbohydrates (final concentration 1%) and bromocresol purple pH indicator was used in determining fermentation reactions. Inocula were taken from unsupplemented THB, and reactions were read daily for as long as 7 days. Except for specimens grown on Mueller arginine, which was overlaid with mineral oil, cultures were incubated in air at 37°C. The strains were serologically grouped by the Lancefield technique (14). Group carbohydrates were extracted from overnight THB cultures supplemented with 0.001% pyridoxal·HCl. Gram stains were performed on overnight broth cultures.

Anaerobic testing. The biochemical tests used in the Anaerobe Section Laboratory were those described in *Laboratory Methods in Anaerobic Bacteriology* by Dowell and Hawkins (4). The colony characteristics and purity of the cultures were tested on CDC anaerobe blood agar (5) incubated in a glove box with an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 for 24 to 48 h. Isolated colonies were inoculated to chopped meat-glucose broth and enriched thioglycollate medium (5). After Gram staining, the isolates were inoculated into differential media as described previously (4). Fermentation of carbohydrates was tested with carbohydrate (CHO) fermentation media (5) containing 0.6% final concentration of carbohydrate and bromothymol blue pH indicator. The isolates were also tested for indole and H_2S production; nitrate reduction; action on milk; hydrolysis of esculin, starch, and gelatin; and production of urease, catalase, lecithinase, lipase, and deoxyribonuclease (4).

Acid metabolic products of the organisms were ether extracted (4) and analyzed with gas-liquid chromatography in a Capco model 700 chromatograph (Clinical Analysis Products Co., Sunnydale, Calif.) equipped with a thermoconductivity detector and Supelco 10% SP1000/1% H_3PO_4 on 100/120 Chromosorb W/AW packing material (Supelco, Inc., Supelco Park, Bellefonte, Pa.). The carrier gas (helium) flow rate was 90 cm^3/min, and the operating temperature of the column was 145°C.

RESULTS

All 25 of the nutritionally variant streptococci recovered from storage grew within 48 h in fluid thioglycollate broth and in THB supplemented with 0.001% pyridoxal·HCl (Table 1). However, growth in the supplemented THB produced noticeably greater turbidity than that in fluid thioglycollate broth. Table 1 also shows that fewer isolates grew in Schaedler broth supplemented with vitamin K and hemin, even less in THB supplemented with 5% defibrinated rabbit blood, and the least in unsupplemented THB. All of the strains grew on conventional blood agar, but grew better when 0.001% pyridoxal·HCl was added. The organisms also grew well on anaerobe blood agar, enriched thioglycollate broth, and chopped meat-glucose medium used in the Anaerobe Section Laboratory (5).

Table 2 contains the results of biochemical tests performed on the streptococcus strains when they were first examined in the Streptococcus Laboratory (CDC). All 25 were identified as viridans streptococci on the basis of Gram

TABLE 1. *Growth of nutritionally variant streptococci growing in five liquid medium systems*

Incubation time (h)	Unsupplemented THB		THB + 0.001% pyridoxal·HCl		THB + 5% defibrinated rabbit blood		Schaedler broth + vitamin K and hemin		Fluid thioglycollate broth	
	No. of strains	Turbidity	No. of strains	Turbidity	No. of strains	Turbidity	No. of strains	Turbidity	No. of strains	Turbidity
24	7	Slight	19	Moderate	10	Slight	14	Moderate	20	Slight
48	13	Moderate	25	Heavy	20	Moderate	21	Moderate	25	Slight

TABLE 2. *Biochemical patterns of 25 nutritionally variant streptococci as originally reported by the CDC Streptococcus Laboratory*[a]

Reaction on:								No. of Strains	Identified as viridans streptococci resembling:
Sucrose	Inulin	Trehalose	Lactose	Raffinose	Melibiose	Esculin	Salicin		
+	−	+	+	+	+	−	+	1	*S. sanguis* II
+	+	+	+	V	V	−	−	9	*S. salivarius*
+	−	+	−	−	−	+	−	2	*S. anginosus-constellatus*
+	+	−	−	V	−	V	−	3	Unidentified
V	−	−	−	−	−	−	−	10	*S. morbillorum*

[a] All strains failed to serogroup (A, B, C, D, F, G), were resistant to bacitracin, and reacted negatively for catalase, starch, hippurate, 6.5% NaCl, methylene blue milk, tellurite, tetrazolium, arginine, sorbitol, mannitol, glycerol, arabinose, 10°C and glucan tests. +, positive reaction; −, negative reaction; V, variable reaction.

reaction and morphology (gram-positive cocci in chains); gamma- or alpha-hemolysis; and negative results were obtained from catalase, serological grouping, 6.5% NaCl tolerance, bile esculin, bile solubility, and optochin sensitivity testing. Biochemical patterns of 22 strains were similar to those of species included in the viridans streptococci (Table 2). Nine were phenotypically similar to *Streptococcus salivarius* except that they could not hydrolyze esculin or produce extracellular polysaccharides. Ten were similar to *S. morbillorum*, two were similar to *S. anginosus-constellatus*, and one was similar to *S. sanguis* biotype II. The biochemical patterns of three strains were different from those of any known *Streptococcus* species in the viridans group. Table 3 lists key biochemical and physiological reactions of the same strains recovered from storage when tested in the CDC Streptococcus Laboratory (heart infusion broth base media) and in the CDC Anaerobe Section Laboratory (CHO base media). When tests were repeated, minor differences in the biochemical reactions of the strains in the Streptococcus Laboratory system resulted in four changes in identification (Table 3). One isolate originally having characteristics similar to those of *S. morbillorum* was reclassified as being more similar to *S. mitis* on the basis that it fermented lactose. Two other isolates which previously were identified as *S. morbillorum* were subsequently able to ferment raffinose, lactose, melibiose, and trehalose and were thus phenotypically closer to *S. sanquis* II. The fourth isolate was reclassified from "unidentified" to resembling *S. salivarius* after the tests were repeated. All other reactions remained unchanged. The biochemical patterns of all 25 isolates remained unchanged when the isolates were retrieved from storage and tested in the Streptococcus Laboratory approximately 2 months after these latter tests.

In the Anaerobe Section Laboratory, with CHO media, all of the *Streptococcus* strains fermented glucose, maltose, and sucrose and did not ferment mannitol, glycerol, rhamnose, and melezitose. The strains did not hydrolyze starch or gelatin; reduce nitrate or iron milk; or produce indole, H_2S, urease, lecithinase, lipase, deoxyribonuclease, or catalase activity and were nonmotile. The major metabolic product of 14 of the isolates was lactic acid. The ratio of lactic acid to acetic acid, based upon peak height, was approximately equal for 10 of the isolates, but 1 isolate of *S. morbillorum* produced an acetic acid peak five times greater than the lactic acid peak.

The overall agreement of 86.5% was obtained for reactions measured in the Streptococcus Laboratory and in the Anaerobe Section Laboratory in certain key tests (sucrose, trehalose,

TABLE 3. *Results of differential tests performed on nutritionally variant streptococci after recovery from storage*[a]

Streptococcus Laboratory identification	Strain no.	Fermentation of:							Esculin hydrolysis	CHO base identifcation
		Sucrose	Trehalose	Inulin	Lactose	Raffinose	Melibiose	Salicin		
S. salivarius	1635	+	+	(+)	+	+	(+)	−	(+)	*S. sanguis II*
S. salivarius	1995	+	+	+	+	+	(+)	−	−	*S. salivarius*
S. salivarius	2426	+	+	+	+	(+)	(+)	−	−	*S. salivarius*
S. salivarius	2459	+	+	+	+	(+)	(+)	−	−	*S. salivarius*
S. salivarius	2420	+	+	(+)	+	−	+	−	−	*S. mitis*
S. salivarius	859	+	+	(+)	(+)	−	(+)	−	−	*S. anginosus*
S. salivarius	1091	+	+	(+)	+	−	(+)	−	−	*S. mitis*
S. salivarius	1848	+	+	(+)	+	−	(+)	−	(−)	*S. mitis*
S. salivarius	1771	+	+	(+)	+	−	−	−	−	*S. mitis*
S. salivarius	1089	+	+	(+)	+	−	−	−	−	*S. mitis*
S. anginosus	1760	+	−	−	−	−	−	+	+	*S. anginosus*
S. anginosus	1761	+	−	−	−	−	−	+	+	*S. anginosus*
S. morbillorum	169	+	−	−	−	−	−	−	(+)	*S. morbillorum*
S. morbillorum	215	+	−	−	−	−	−	−	−	*S. morbillorum*
S. morbillorum	371	+	−	−	−	−	−	−	(+)	*S. morbillorum*
S. morbillorum	2032	+	−	−	−	−	−	−	−	*S. morbillorum*
S. morbillorum	2449	+	−	−	−	−	−	−	−	*S. morbillorum*
S. morbillorum	744	+	−	−	−	−	−	−	−	*S. morbillorum*
S. mrobillorum	920	(−)	−	−	−	−	−	−	+	*S. morbillorum*
S. sanguis II	2460	+	+	−	+	+	+	−	−	*S. sanguis II*
S. sanguis II	1076	+	+	−	(+)	+	(+)	(+)	−	Unidentified
S. sanguis II	2458	+	+	−	+	+	(+)	−	−	*S. sanguis II*
S. mitis	743	+	−	−	(+)	−	−	−	−	*S. morbillorum*
Unidentified	1859	+	−	+	−	(+)	−	−	(+)	Unidentified
Unidentified	1061	+	−	+	−	−	−	−	−	Unidentified

[a] ( ), Different reaction obtained in Anaerobe Section with CHO fermentation media; +, Positive results; −, negative results.

inulin, lactose, raffinose, melibiose, salicin, esculin; Table 3) used in identification. Most discrepancies between the two systems resulted from variations in the fermentation of melibiose, raffinose, and inulin and in the hydrolysis of esculin.

DISCUSSION

The involvement of the alpha-hemolytic streptococci in bacterial endocarditis has been extensively documented, with incidences as high as 94% having been reported (3, 12, 13). A recent review at New York's Columbia-Presbyterian Medical Center of 101 endocarditis cases diagnosed clinically between 1968 and 1973 indicated that streptococci accounted for 62% of the cases (32% of which were caused by viridans streptococci). Blood cultures from 12 of the patients were negative, but antibiotics were administered before hospital admission to 2 of them (9). No satelliting organisms or L-forms were detected. It has been recommended that bacteriologists be prepared to handle negative blood cultures in situations involving endocarditis by using thioglycollate broth or a cysteine-supplemented broth and the satellite test in detecting nutritionally deficient organisms (15). On the basis of our study of the optimal growth conditions for the nutritionally deficient streptococci, we concluded that pyridoxal·HCl is the best growth supplement for these organisms in broth media. The optimal concentration of pyridoxal to use with these microorganisms was determined to be 0.001% (2). Although these streptococci grow readily on solid infusion agar supplemented with 5% animal blood, they grow better in the presence of 0.001% pyridoxal·HCl in the agar medium. Fluid thioglycollate broth also supports the growth of the organisms, but turbidity was considerably less than that obtained in supplemented Todd-Hewitt broth. Although continual medium passage resulted in adaptation of all the nutritional variants tested, none grew upon primary isolation without supplementing either pyridoxal or a thiol compound to broth or without streaking *S. epidermidis* to seeded plates. This apparently is a characteristic common to all nutritionally variant streptococci and should be considered during attempted isolation of these organisms in vivo. When four of these organisms adapted to standard streptococcal media, their biochemical patterns changed as well (Tables 2 and 3). The reason for these changes was not determined.

There were also a number of differences in the reactions obtained with certain key differential tests in the CHO base media used in the Anaerobe Section Laboratory and with those in the heart infusion broth base media used in the Streptococcus Laboratory (Table 3). Fermentation of the carbohydrates in both basal media was determined by pH indicator color changes. Whereas the heart infusion broth base carbohydrate fermentations and other biochemicals used in the Streptococcus Laboratory are incubated in air, the CHO base biochemicals were incubated anaerobically. Other incubation conditions were the same for both systems. Since the eight differential tests listed in Table 3 are included among the rigid criteria for identifying the viridans streptococci as to species (6), only minor differences in the biochemical patterns between the two systems resulted in different identifications. Consequently, 7 of 10 isolates identified as *S. salivarius* in the heart infusion broth base system were identified as other viridans species in the CHO base system. Likewise, one isolate of *S. sanguis II* was characteristically unsimilar to any viridans species when tested in CHO base biochemicals. Strain 743 (*S. mitis*) was identified as *S. morbillorum* since it did not ferment lactose in the CHO base system. Even though the biochemical patterns obtained in the heart infusion broth base system remained stable for at least 2 months after the organisms adapted, no assurances can be made that their phenotypic characteristics, and perhaps identifications, will not vary in the future. This phenomenon is not peculiar to the nutritionally variant streptococci, but has also been observed with other isolates retrieved from storage in the Streptococcus Laboratory. These results further emphasize the point recently made by Stargel et al. (17) that variations in biochemical reactions are to be expected when differential tests with widely differing basal media, growth conditions, and pH indicators are used. They also pointed out that regardless of the system used in identifying bacterial isolates, the differential tables used should be based on data obtained with the same differential media.

The diversity of physiological patterns obtained with the nutritionally variant isolates suggests that at least six different species of viridans streptococci are represented. It does not appear that a new species has emerged among these strains. Instead, loss of key enzymes in certain metabolic pathways may result in phenotypes divergent from those of known species.

The streptococci are included among the homolactic bacteria, because lactic acid is the major product from the fermentation of carbohydrate when peptone–yeast extract–glucose is used as the substrate. However, factors such as composition of the medium, carbohydrate

source, pH, incubation conditions, and duration of incubation have been shown to influence the production of lactic acid as well as acetic and formic acids (11, 16). Lactic acid was a major product of all of the nutritionally variant streptococci in our study. However, 10 isolates produced approximately the same amounts of acetic and lactic acid, and gas-liquid chromatography results indicated that one strain produced more acetic than lactic acid. These results may be affected by the fastidious nature of the organisms. It is known that varying the amino acid concentration in substrates causes alterations in metabolic products (G. L. Lombard, personal communication). The apparent deficiency of pyridoxal among nutritionally variant streptococci may therefore alter their metabolites since pyridoxal is a required coenzyme in the synthetic pathways of several amino acids.

The relationship of streptococcal L-forms to the nutritionally variant streptococci has not been clearly defined. There is also very little evidence of a causal relationship between recovery of nutritionally variant streptococci from patients with systemic disease and treatment with inhibitors of cell wall synthesis such as penicillins and cephalosporins. However, George suggested that the requirement of the organisms for vitamin B_6 (or its analogs, including pyridoxal) may be related to a deficiency in processes other than cell wall formation (10). Now that we have demonstrated that pyridoxal·HCl was a growth enhancer rather than a growth requirement for the adapted nutritionally variant streptococci, further studies of the metabolic deficiencies of these organisms must be done to clarify the relationships between nutritionally variant streptococci and L-forms.

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