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Detection of Diacetyl (Caramel Odor) in Presumptive Identification of the "*Streptococcus milleri*" Group

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The caramel odor associated with the "*Streptococcus milleri*" group was shown to be attributable to the formation of the metabolite diacetyl. Levels of diacetyl in the 22- to 200-mg/liter range were produced by 68 strains of the "*S. milleri*" group; apart from one strain of *Streptococcus mutans*, all 92 other strains of streptococci belonging to 12 species produced <13 mg of diacetyl per liter. Quantitation of diacetyl levels from cultures of streptococci is suggested as a rapid presumptive test for the "*S. milleri*" group.

Over the past decade there has been increased awareness of the importance of the "*Streptococcus milleri*" group as a pathogen (1, 4). Though this group of streptococci has been classified into a diverse number of species, the findings of Coykendall et al. (2) suggested that the group was sufficiently related to be all called *Streptococcus anginosus*. However, further studies by Whiley and Beighton have resulted in the subdivision of this group into three separate species—*S. anginosus*, *Streptococcus intermedius*, and *Streptococcus constellatus* (5). Some authors have noted that agar cultures of the "*S. milleri*" group produce a characteristic smell that has been described as caramel-like (3). It had been suggested that the odor appeared to be almost diagnostic for this species. An attempt was therefore made to determine the metabolite responsible for this odor and its potential for presumptive identification of this species.

Sixty-eight strains of the "*S. milleri*" group isolated in the Microbiology Laboratory from patients at Dunedin Hospital were included in the investigation. Caramel odor was not used as a criterion for identification of strains; all were identified by using the API Rapid Strep system (Analytab Products, Vercieu, France). Also included were 10 strains of *Streptococcus pyogenes*, 10 strains of "*Streptococcus equisimilis*," 10 strains of Lancefield group G streptococci, and 8 strains of *Streptococcus agalactiae*. These were characterized by determining the Lancefield group by using a Streptococcal Grouping Kit (Oxoid Limited). A further 55 strains of streptococci were supplied by John Tagg of Otago University. These consisted of 9 strains of *Streptococcus mutans*, 12 strains of *Streptococcus salivarius*, 10 strains of *Streptococcus sanguis*, 3 strains of *Streptococcus sobrinus*, 7 strains of *Streptococcus gordonii*, 5 strains of *Streptococcus rattus*, 3 strains of *Streptococcus oralis*, and 6 strains of *Streptococcus uberis*.

Gas chromatographic analysis was performed on a Pye Unicam series 204 gas chromatograph using a flame ionization detector. A capillary column (10 m by 0.53 mm) coated with SUPEROX FA to a thickness of 1.2 μm was used (Alltech Associates, Inc.). The chromatograph oven was run isothermally at 60°C, and the injection and detector port ovens were set at 100°C. The nitrogen carrier gas flow was 5 ml/min, the hydrogen flow was 20 ml/min, and the air flow was 500 ml/min.

Six strains of the "*S. milleri*" group, a strain each of *S. pyogenes* and Lancefield group G streptococcus, and three strains of *S. agalactiae* were examined in an attempt to characterize a metabolite unique to the "*S. milleri*" group. All strains were plated onto Columbia blood (5% sheep blood) agar and incubated for 18 h at 37°C. Then 2-cm^2 portions of agar were placed into 5-ml vials with Teflon-lined septae. These were heated in a 56°C water bath for 30 min to allow volatile components to enter the gaseous phase. A 0.5-ml fraction of the headspace gas was removed with a 1-ml gas-tight syringe and analyzed by the gas chromatograph. The chromatograms of the "*S. milleri*" strains all revealed a unique peak with a retention time of approximately 90 s. None of the other streptococcal strains studied produced such a peak (Fig. 1).

To provide sufficient quantities of the metabolite for analysis, 1 liter of Todd-Hewitt broth was inoculated with approximately 10^5 organisms of the type strain of *S. anginosus* (NTCC 8037). This was incubated for 18 h at 37°C while being shaken at a speed of 100 oscillations per min. This broth was then subjected to fractional distillation, and the fraction containing compounds with boiling points between 50 and 90°C was collected in a 100-ml round-bottomed flask immersed in dry ice. The volatile components were dissolved in 0.5 ml of octanol. The concentrated volatile mixture was then analyzed with a Kratos MS 80 RFA combined gas chromatograph-mass spectrograph. The components were thereby separated and identified by comparing the unique spectra with those in the resident computerized library. The unique compound was identified as being diacetyl. The odor of an aqueous solution of diacetyl was noted as being identical to the caramel odor detectable in "*S. milleri*" group cultures.

To determine the relative quantity of diacetyl produced by the different species of streptococci included in the study, a 2-mm loopful of each strain was harvested from blood agar cultures and inoculated into 1.5-ml volumes of Todd-Hewitt broth (Difco Laboratories). The broths were incubated for 4 h at 37°C in screw-cap glass bottles (25 by 80 mm), and a 1.0-ml aliquot was transferred to a 4-ml screw-cap septate vial. The vials were placed in a 60°C water bath for 30 min, and the vials were shaken for 10 s at 10-min intervals to allow the volatile components to equilibrate in the headspace gas. A series of standards of aqueous solutions of diacetyl was

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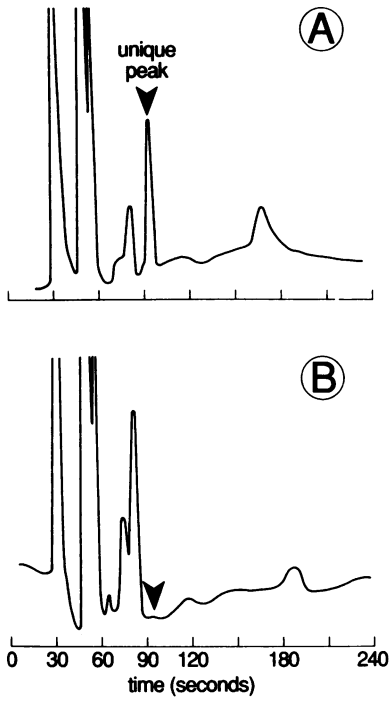

FIG. 1. Headspace chromatogram of blood agar cultures of the "*S. milleri*" group (A) and *S. pyogenes* (B).

similarly prepared. A 1.0-ml sample of the headspace gas from each vial was analyzed by gas chromatography as for the blood agar samples, and the peak area of the diacetyl was measured. Both the test samples and the standards were tested in duplicate. A standard curve was plotted, and the quantity of diacetyl produced by each organism was determined. Under the specified conditions, all 68 strains of the "*S. milleri*" group studied produced significant quantities of diacetyl ranging from 22 to 200 mg/liter (Table 1).

Diacetyl levels obtained from repeated testing of a series of individual strains revealed that reproducibility was within 25%. While one strain of *S. mutans* produced a level of 180 mg/liter, the remainder of the non-"*S. milleri*" strains tested produced levels that ranged from <1 to 12 mg/liter. A pronounced caramel odor could be detected from agar cultures of the *S. mutans* strain that produced high levels of diacetyl. A limited study of 26 strains of the "*S. milleri*" group that were differentiated into the three distinct species (*S. anginosus*, *S. intermedius*, and *S. constellatus*) by methods described by Whiley et al. (6) revealed no significant difference in diacetyl production between the species. It has been suggested that the caramel odor produced by strains of the "*S. milleri*" group could be used as a presumptive

TABLE 1. Diacetyl production by various streptococcal species

Species (no. of strains)	No. of strains producing indicated amt of diaceyl (mg/liter)			
	<1	1–10	10–20	>20
S. mutans (9)	3	5	0	1
S. gordonii (7)	5	2	0	0
S. sanguis (10)	10	0	0	0
S. rattus (5)	5	0	0	0
S. sobrinus (3)	2	1	0	0
S. oralis (3)	3	0	0	0
S. salivarius (12)	6	5	1	0
S. uberis (6)	6	0	0	0
S. pyogenes (10)	8	2	0	0
S. agalactiae (8)	4	4	0	0
S. "equisimilis" (10)	10	0	0	0
Lancefield group G streptococci (10)	8	2	0	0
"*S. milleri*" group (68)	0	0	0	68

identification test (2). However a number of "*S. milleri*" group strains included in the present study which appeared devoid of an obvious caramel odor in culture revealed diacetyl levels ranging from 22 to 45 mg/liter by gas chromatography.

These results suggest that under the conditions described, streptococcal strains that produce diacetyl levels of >15 mg/liter could be presumptively identified as belonging to the "*S. milleri*" group. The identification of the metabolite responsible for the caramel odor and the ability to quantitate it by gas chromatographic analysis increase the sensitivity and remove the subjectivity inherent in the olfactory method of detection.

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