Title: The *Streptococcus milleri* population of a cystic fibrosis clinic reveals patient specificity and intra-species diversity

Authors: Christopher D. Sibley*, Kristen A. Sibley*, Tara A. Leong, Margot E. Grinwis, Michael D. Parkins, Harvey R. Rabin and Michael G. Surette

* these authors contributed equally to this work

Affiliation: 1Department of Microbiology and Infectious Diseases; 2Department of Medicine; 3Adult Cystic Fibrosis Clinic; 4Department of Biochemistry and Molecular Biology, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1.

Corresponding Author: Michael G. Surette, Department of Microbiology and Infectious Diseases, University of Calgary, Faculty of Medicine, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Tel: 403-220-2744, Fax: 403-270-2772, surette@ucalgary.ca
Abstract

The genetic relatedness of *Streptococcus milleri* group isolates from the airways of cystic fibrosis patients was determined by using pulsed-field gel electrophoresis. This study reveals no evidence for patient-to-patient transmission in our patient population; however, within individual patients, complex inter- and intra-species diversity and dynamics can be observed.

Article

In addition to their role in purulent infections (3, 7, 11), the *Streptococcus milleri* Group (SMG, also known as the *S. anginosus* group), comprised of *S. constellatus*, *S. intermedius* and *S. anginosus*, have emerged as clinically relevant in chronic airway infection in cystic fibrosis (CF) patients and have been implicated as an etiologic agent of pulmonary exacerbation (6, 10, 13, 14). We have recently described the isolation of a large number of SMG strains from a cohort of CF patients by using the semi-selective medium McKay agar (12). This collection of SMG respiratory isolates was not recovered by conventional CF microbiology and enabled us to characterize the phenotypic properties of airway isolates and compare them to invasive strains (5). These results, in combination with analysis of the nucleotide sequence of the 16S rRNA gene of these strains, revealed clusters of isolates that included both CF and invasive isolates with indistinguishable phenotypic characteristics (5, 9).

In this study, we evaluated whether patient-to-patient transmission was occurring in our CF patient cohort. The molecular epidemiological relationship of the SMG isolates was determined by using pulsed-field gel electrophoresis (PFGE) (4, 8, 15).
PFGE was performed by modification of a protocol described by Bartie et al. (2). Isolates were cultured at 37°C for 48 h on brain heart infusion agar supplemented with colistin sulfate (10 µg/ml) and oxolinic acid (5 µg/ml) under anaerobic conditions. Cells were harvested and suspended to 20% transmittance (600 nm) in 100 mM Tris-HCl buffer (pH 7.6). Mutanolysin (100 U; Sigma Aldrich, St. Louis, MO) was added to 500 µl of cell suspension before an equal volume of molten 1% SeaKem Gold agarose (Lonza, Rockland, ME). Plugs were cast at room temperature then transferred to 1.5 ml of lysis solution (0.25M EDTA [pH 9.0], 0.5% Brij 58, 2 g/l sodium deoxycholate, 5 g/l lauroyl sarcosine, 100 U/ml mutanolysin) and incubated at 37°C for 2 h. The lysis solution was replaced with 1.5 ml ESP solution (0.25M EDTA [pH 9.5], 1% sodium lauroyl sarcosine, 0.5 mg/ml proteinase K) and incubated at 55°C for 2 h. Plugs were rinsed with 1 ml of distilled water then washed for 10 min intervals, once with distilled water and three times with 1X TE at room temperature. For the restriction digestion, plugs were pre-incubated in 300 µl of 1X reaction buffer (Invitrogen, Carlsbad, CA) at room temperature for 15 min and then replaced with fresh 1X reaction buffer supplemented with 90U of SmaI or ApaI (New England BioLabs, Beverly, MA). SmaI and ApaI digestion occurred at room temperature and 31°C respectively for 4 h. Following digestion, the plugs were briefly rinsed twice in 1X TE. Following a 5 min wash in 1X TE, plugs were loaded into a 1% SeaKem Gold agarose gel, prepared in 0.5X TBE (1X TBE is 89 mM Tris HCl [pH 7.4], 89 mM boric acid, 25 mM EDTA [pH 8.0]). The following parameters were used: gradient, 6.0 V/cm; run time, 22 h; included angle, 120°; initial switch time, 10 s; and final switch time, 35 s at 14°C.
A database of the PFGE profiles was developed with BioNumerics software (Applied Maths, Saint-Martens-Latem, Belgium). Dendrograms were generated using the unweighted pair group method using average linkages with a 1.0% position tolerance and the Dice coefficient correlation. The threshold required to justify analysis with an additional restriction enzyme was 90%.

Fifty-nine unique profiles were observed from 76 SMG isolates cultured from expectorated sputum of 40 patients (Fig. 1). Such high genetic heterogeneity has been previously observed in the SMG (1). Four isolates from three patients were refractory to PFGE profiling.

Notably, only two isolates (M316 and C266) recovered from different patients clustered above 90% identity by Smal profiling (Fig. 1). To further resolve if these isolates might represent patient-to-patient transmission, we tested all available S. constellatus isolates recovered longitudinally from these patients (two and three isolates, respectively) with a secondary restriction enzyme, Apal (Fig. 2). This analysis revealed that these strains were in fact more genetically divergent and clustered at less than 80% identity. Given that these strains represent distinct PFGE profiles, and these two patients had not attended clinic on the same day or been in-patients at the same time, these appear to represent different strains.

PFGE revealed a diverse SMG population. Multiple SMG isolates per patient (collected either longitudinally or cross-sectionally) were analyzed from a total of 15 patients. Multiple PFGE profiles were detected in 11 patients (Fig. 1). Four of the patients with multiple SMG strains revealed intra-species diversity; as many as three distinct strains of the same species were represented in a single patient (Fig. 1).
Isolates were available for longitudinal analysis in seven of the 11 patients (63.6%) with multiple SMG strains present. In six of these cases (85.7%) at least one of the strains was isolated on at least two occasions. It was possible to investigate longitudinal isolates by PFGE in four additional patients. In total, the same isolate was recovered at multiple time points in nine of the 11 patients (81.8%) analyzed longitudinally. As previously reported, this supports that chronic colonization is common (2, 13), although it may not always be the case.

The complex dynamics of SMG populations within certain patients was noteworthy. Three examples of patients are shown in Figure 3 that illustrate both the diversity within the SMG at the species and strain level, as well as the complex population dynamics that can occur over time.

We have determined that SMG strains in our CF patient population are patient-specific and we have no evidence for the occurrence of patient-to-patient transmission. Intriguingly, this study reveals an unexpected level of complexity in SMG populations detected in the sputum of certain CF patients. Moreover, it is important to consider that the intra-species diversity we observe may still under-represent the true population richness due to limited sampling. To fully appreciate the extent of genetic heterogeneity in chronic infections deep sampling may be required. As we continue to investigate the CF airway microbiome, strain-level diversity and dynamics may be integral to developing predictive models of clinical perturbation.

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References


Figure Legends

Figure 1. The relationship between all of the unique PFGE profiles generated with *SmaI* from the SMG isolates used in this study. Chronically colonizing strains (recovered from the same patient on multiple occasions) are indicated with an asterisk. Patients with multiple SMG strains are represented as vertical dotted lines; SMG isolates of different species (*S. anginosus* (ANG), *S. intermedius* (INT) and *S. constellatus* (CON)) are shown with a solid circle. In patients where intra-species diversity was observed, strains of the same species are depicted with solid squares. Strain and species are indicated on the left and right of the gel profiles, respectively.

Figure 2. The *ApaI* and *SmaI* fingerprints of multiple isolates (recovered at different times as shown in days) from two patients with closely related *SmaI* profiles reveals that the strains are genetically different enough to rule out patient-to-patient transmission. The profiles indicated with the asterisk are also represented in Fig. 1.

Figure 3. The genetic relatedness of strains recovered from three patient examples (A, B and C) that demonstrate inter- and intra-species diversity. Population dynamics of the SMG strains from longitudinal sampling are shown next to the corresponding PFGE profile. Strains of the same or different species are depicted with solid squares or circles, respectively. Strain and species are indicated on the left and right of the gel profiles, respectively. The profiles indicated with the asterisk are also represented in Fig. 1.