Safety Assessment of the Oral Cavity Probiotic *Streptococcus salivarius* K12

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***Streptococcus salivarius* is a prominent member of the oral microbiota and has excellent potential for use as a probiotic targeting the oral cavity. In this report we document safety data relating to *S. salivarius* K12, including assessment of its antibiogram, metabolic profiles, and virulence determinants, and we examine the microbial composition of saliva following the dosing of subjects with K12.**

Certain lactic acid bacteria (LAB) have had a long history of consumption by humans, either as probiotics or in traditional foods. Proposals for the use of nontraditional species in humans generally evoke greater concern about potential adverse effects than proposals for LAB probiotics (11, 12). Nevertheless, even species generally regarded as safe and with long histories of application can still potentially cause infection in humans. Recent indications are that some of the more exciting new probiotic developments will include a shift in focus toward strains having both their origins and primary mucosal targets in tissues other than the intestinal tract (5).

Although there have been some attempts to use intestinally derived bacteria such as lactobacilli for oral cavity probiotics, it appears more likely that bacteria isolated directly from the oral microbiota will be efficacious for such purposes (5). *Streptococcus salivarius* K12 (isolated from the saliva of a healthy child) is a probiotic intended for use in the oral cavity. Strain K12 has had a 5-year history of commercial application as a probiotic in New Zealand, with approximately 150,000 doses administered to date. Its in vitro antimicrobial activity against *Streptococcus pyogenes* and various bacterial species incriminated in the etiology of halitosis appears to be due to the production of lantibiotic bacteriocins (14, 18, 21). *Streptococcus salivarius* is a prominent member of the oral microbiota of "healthy" humans and is closely related to *Streptococcus thermophilus* (3), a benign organism used in the manufacture of yogurt. *Streptococcus salivarius* is known to be a pioneer colonizer of infants, who typically acquire it from their mothers shortly after birth (7, 10, 19). As with lactobacilli, there have been occasional reports of infections involving *S. salivarius*, though their occurrence (even in adverse medical conditions) is extremely low (1, 2, 6, 8, 16, 20, 23).

What safety considerations should apply to a probiotic intended for application in the oral cavity? Many of the requirements for intestinal probiotics are relevant here, for example, whether the bacterium exhibits (i) antibiotic resistance, (ii) metabolic activities potentially adversely affecting the host, or (iii) inhibitory activity against other commensal microorgan-

isms. Consideration should also be given to the evolutionary origins of probiotic candidates as an indicator of the potential for them to carry particular virulence determinants. For example, the genus *Streptococcus* includes many species that are largely commensals of the mucosal membranes of the upper respiratory tract, and some species commonly cause disease.

The antibiograms of three samples of strain K12 were tested by the antibiotic disk sensitivity method (conducted according to CLSI [formerly NCCLS] standards) to determine whether they exhibited any differences in profile. Strains tested were (i) the original isolate (K12-J89), stored at −70°C for 15 years, (ii) a laboratory stock culture (K12-Lab) that had been subcultured every 2 weeks for 3 years, and (iii) a commercially prepared batch (K12-BN21) of freeze-dried cells. The antibiograms of the K12 isolates did not differ following long-term storage, recurrent in vitro propagation, or commercial lyophilization (Table 1). *Streptococcus salivarius* K12 was assessed to be moderately resistant to both gentamicin and ofloxacin. Eight additional *S. salivarius* isolates from different individuals were also tested for sensitivity to gentamicin and ofloxacin to help determine the level of resistance to these antibiotics in the general *S. salivarius* population. Each displayed moderate levels of resistance to gentamicin and ofloxacin, similar to that of strain K12 (Table 1). Thus, *S. salivarius* K12 is sensitive to a variety of commonly utilized antibiotics, including several that are routinely used for the control of upper respiratory tract infections. The low levels of gentamicin and ofloxacin resis-

TABLE 1. Antibiotic disk sensitivities of *S. salivarius* isolates

| Antibiotic (concn [μg]) | Inhibition zone size (mm) for: | | | | | | | | | | |
| | Strain K12 lineage | | | HD | ToveR | #6 | K30 | HA | HB | HC | K26R |
	K12-J89[a]	K12-Lab[b]	K12-BN21[c]								
Penicillin (10)	34	34	39	34	29	26	26	26	27	27	28
Amoxicillin (10)	35	32	35	35	28	26	29	26	26	28	27
Ofloxacin (5)	18[d]	18[d]	15[d]	18[d]	19[d]	17[d]	16[d]	16[d]	18[d]	16[d]	18[d]
Tetracycline (30)	28	27	26	28	27	27	22	27	28	28	27
Erythromycin (15)	30	30	31	30	28	29	26	28	29	27	27
Gentamicin (10)	15[d]	14[d]	14[d]	15[d]	15[d]	16[d]	14[d]	12[d]	12[d]	12[d]	14[d]
Clindamycin (2)	29	26	28	29	28	26	26	29	30	28	25

[a] Original isolate.
[b] Routinely subcultured isolate.
[c] Isolate from commercial batch.
[d] Moderately resistant.

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TABLE 2. API 20 Strep and API 50CH positive reactions for
Streptococcus salivarius K12 cultures

Test	Reaction of:		
	K12-J89[a]	K12-Lab[b]	K12-BN21[c]
API 20 Strep[d]			
Acetoin production	+	+	+
β-Glucosidase	+	+	+
Alkaline phosphatase	+	+	+
Leucine aminopeptidase	+	+	+
D-Lactose	+	+	+
D-Trehalose	+	+	+
Inulin	+	+	+
D-Raffinose	+	+	+
API 50CH			
D-Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
N-Acetylglucosamine	+	+	+
Arbutine	+	+	+
Salicin	+	+	+
D-Cellobiose	+	+	+
D-Maltose	+	+	+
D-Lactose	+	+	+
D-Saccharose	+	+	+
D-Trehalose	+	+	+
Inulin	+	+	+
D-Raffinose	+	+	+
D-Tagatose	+	+	+

[a] Original isolate.
[b] Routinely subcultured isolate.
[c] Isolate from commercial batch.
[d] The API 20 Strep code for all three isolates was 5060470.

tance in strain K12 were similar to those of a series of natural *S. salivarius* isolates, indicating that they are intrinsic resistances.

In order to determine the metabolic profile of strain K12 and its stability, the API 20 Strep and API 50CH systems (bioMérieux, Marcy-l'Etoile, France) were utilized. None of the fermentation or enzymatic reactions of *S. salivarius* K12 are indicative of deleterious effects for the human host (Table 2). Additionally, the metabolic profiles given by strain K12 following either recurrent propagation or commercial processing were identical to that of the original isolate, indicating that the
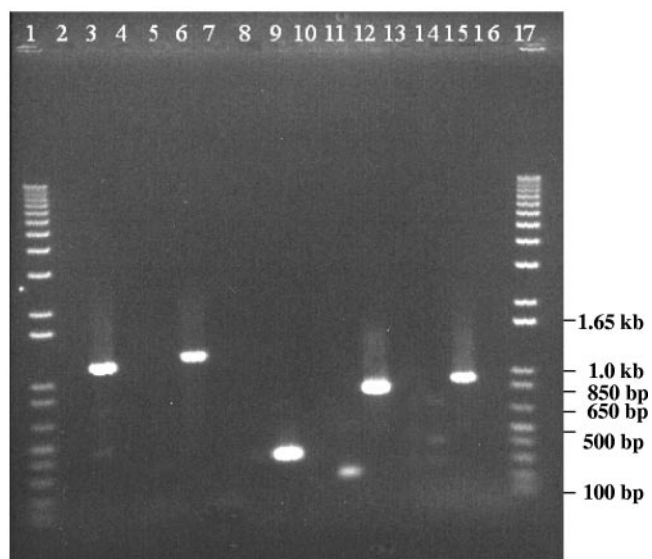

FIG. 1. *Streptococcus salivarius* K12 (even-numbered lanes) and *S. pyogenes* SF370 (odd-numbered lanes) amplification products resulting from PCR using specific primers for the *sagA* (lanes 2 and 3), *scpA* (lanes 5 and 6), *smez-2* (lanes 8 and 9), *speB* (lanes 11 and 12), and *emm* (lanes 14 and 15) genes. Lanes 1 and 17, 1-kb marker (Gibco).

phenotypic expression of metabolites and fermentation pathways represents stable characteristics of this strain. The ability of *S. salivarius* K12 to lyse red blood cells was tested on three media: (i) human blood agar (BaCa, consisting of Columbia agar base with 5% [vol/vol] human blood; Fort Richard Laboratories, New Zealand), (ii) sheep blood agar (Columbia agar base with 5% [vol/vol] defibrinated sheep blood), and (iii) buffered (pH 7.5) CNA-P agar (Difco) with 5% defibrinated sheep blood (9). In each case, no hemolytic activity was detected.

For the detection of known streptococcal virulence determinants, chromosomal DNA was extracted from cultures of *S. salivarius* strain K12 and *S. pyogenes* strain SF370 (M-serotype 1, genome strain) using the DNeasy tissue kit (QIAGEN, Valencia, CA). The presence of streptococcal virulence genes in SF370 and K12 was assessed using the specific primers described in Table 3. Amplicons from *S. pyogenes* strain SF370

TABLE 3. Primers used to amplify streptococcal virulence genes

| Virulence determinant	Gene designation	Primer name	Primer sequence (5′–3′)	Amplicon size (bp)	Reference
Streptolysin S	*sagA*	sagA Fwd sagB Rev	ATTGAGCTAGCCTTGTCCTTGT GTATTCCGCAAAATCTCTAACG	1,164	This study
C5a peptidase	*scpA*	scpA Fwd scpA Rev	CGGGTATCATGGGACTGTTGC TTGCCGATGTTGCGACTTC	1,259	This study
SMEZ-2	*smez-2*	smez-2 Fwd smez-2 Rev	GGACGAATATGCAGCCAATGA GTATGAAAAACCAGTCTACCAC	332	This study
SPE-B	*speB*	speB Fwd speB Rev	TGACGCTAACGGTAAAGAAAACA GCCGCCACCAGTACCAAGAGC	819	This study
M-protein	*emm*	M-all Fwd M-all Rev	TATTSGCTTAGAAAATTAA GCAAGTTCTTCAGCTTGTTT	961	13
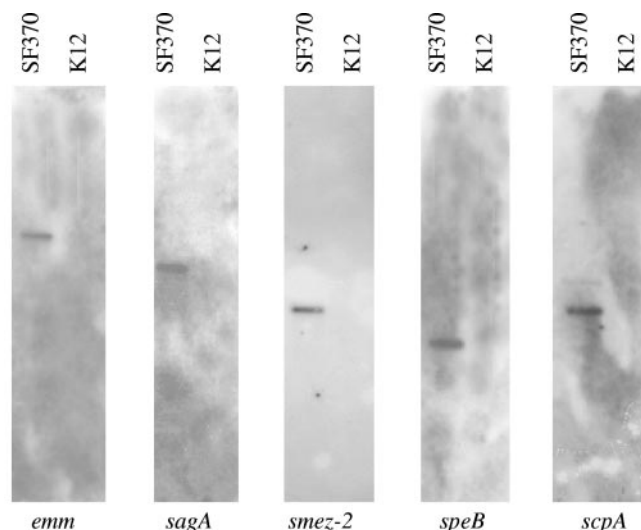
FIG. 2. Autoradiographs of Southern blots hybridized with amplicons of the different streptococcal virulence factors (given at the bottom). Lanes containing HindIII-digested DNA from *S. pyogenes* SF370 are labeled SF370. Lanes containing HindIII-digested DNA from *S. salivarius* strain K12 are labeled K12.

TABLE 5. Detection of specific microorganisms in saliva of individuals prior to and in the days following dosing with *Streptococcus salivarius* K12[a]

Organism(s)	No. of subjects in which the indicated class of microbe was detected/total no. of subjects at the following sampling time:					Maximum CFU/ml detected in any single sample
	Predosing	Day 3	Day 7	Day 14	Day 28	
S. salivarius K12-like BLIS[c] profile	2/14	13/14	12/14	9/14	4/14	NA[d]
S. salivarius K12-like BLIS profile >50%	2/14	7/14	7/14	3/14	1/14	NA
S. mutans[b]	9/14	11/14	9/14	11/14	9/13	6.3e5
S. mutans >1e4 CFU/ml	4/14	4/14	4/14	5/14	4/14	NA
S. mutans >1e5 CFU/ml	2/14	2/14	1/14	1/14	1/14	NA
Lactobacilli	6/14	5/14	4/14	7/14	2/14	2.8e5
Candida	1/14	ND[e]	1/14	1/14	1/14	4.4e4
Coliforms	1/14	2/14	0/14	1/14	1/14	140
Pseudomonas	11/14	8/14	10/14	9/14	5/14	100
Staphylococcus aureus	3/14	4/14	1/14	2/14	2/14	1,000

[a] Subjects included 4 males and 10 females; mean age, 19 years.
[b] P values for time point differences for *S. mutans* counts were not significant (>0.5 by nonparametric analysis of variance).
[c] BLIS, bacteriocin-like inhibitory substance.
[d] NA, not applicable.
[e] ND, not determined.

DNA were labeled with digoxigenin and then used to probe Southern blots resulting from HindIII digestion of K12 and SF370 chromosomal DNA. None of the selected virulence factor genes were detected in strain K12 by PCR or Southern hybridization (Fig. 1 and 2). Unfortunately, there is as yet no genome sequence available for *S. salivarius* to facilitate an in-depth bioinformatics analysis for other potential virulence genes. However, the annotated genome sequence of the closely related organism *S. thermophilus* was recently published, and genes demonstrated in other species to be involved in virulence were either nonfunctional or absent in *S. thermophilus* (4). Preliminary work in our laboratory has shown that *S. salivarius* K12 has an *sbcD* homologue similar to that in *S. thermophilus*. An interesting difference between streptococci considered pathogenic and dairy streptococci is the presence of *sbc* genes in the latter. These products reduce the efficiency of recombination, effectively stabilizing the genome (4).

A study approved by the Otago Ethics Committee was conducted to determine whether the use of the K12 strain by humans altered the composition of the oral microbiota. Saliva samples were collected from 14 individuals 24 h prior to the commencement of the colonization protocol and periodically during the study. On the following day, each subject brushed his or her teeth and rinsed with 10 ml of 0.2% chlorhexidine

gluconate to reduce the population levels of existing oral microbiota. At 2-h intervals for 8 h, the subjects sucked a lozenge containing ca. 1×10^9 CFU of *S. salivarius* K12 (BLIS K12 ThroatGuard). This protocol was repeated on days 2 and 3. No adverse symptoms were reported by any of the subjects. Microbial populations in the saliva specimens were evaluated. Saline dilutions were plated in duplicate on the following media: Mitis-Salivarius agar (Difco) (for *S. salivarius*); CHROMagar Candida, CHROMagar ECC (for *Escherichia coli* and coliforms), and CHROMagar *Staph aureus* (all from CHROMagar Microbiology, Paris, France); *Pseudomonas* isolation medium (Fort Richard Laboratories); TSYCSB selective medium (for *Streptococcus mutans*) (22); and BaCa. The majority of pathogens and opportunistic microorganisms tested for in the saliva were those suggested for the assessment of adverse effects of chemotherapy on the oral microbiota (15). Total counts of *Streptococcus salivarius* and facultatively anaerobic bacteria remained stable throughout the study (Table 4). Examination of the saliva of subjects dosed with *S. salivarius* K12 for 3 days indicated that there was no overt change in its microbial composition. The bacteriocin-like inhibitory substance activity of representative *S. salivarius* isolates was determined as described previously

TABLE 4. Counts of facultatively anaerobic bacteria and *S. salivarius* in saliva of individuals prior to and in the days following dosing with *S. salivarius* K12[a]

Organism(s)	Mean CFU/ml (SD) at the following time of sampling:					Maximum CFU/ml detected in any single sample
	Predosing	Day 3	Day 7	Day 14	Day 28	
Facultatively anaerobic bacteria	3.11e7 (2.4e7)	3.09e7 (2.1e7)	3.98e7 (2.3e7)	3.93e7 (2.9e7)	3.32e7 (2.1e7)	1.1e8
S. salivarius	1.54e7 (2.4e7)	7.80e6 (1.3e7)	1.33e7 (1.1e7)	6.58e6 (5.9e6)	7.2e6 (7.1e6)	7.7e7

[a] Subjects included 4 males and 10 females; mean age, 19 years. P values for time point differences for counts of facultatively anaerobic bacteria and *S. salivarius* were not significant (>0.5 by nonparametric analysis of variance).

(17). Two subjects had *S. salivarius* organisms in their oral cavities that exhibited bacteriocin profiles similar to that of strain K12 prior to the taking of the course of K12 lozenges. After 2 days of lozenge taking, 13 of the 14 subjects had *S. salivarius* populations in which more than 1% exhibited strain K12-like inhibitory activity, but by day 28, this was reduced to only 4 subjects (Table 5). These bacteriocin-producing cell lines appeared in some cases to persist in the oral cavity for more than 1 month after the completion of the course. *Streptococcus salivarius* K12 isolates obtained from the saliva of 5 subjects at day 14 were tested by API 20 Strep and 50CH kits, and no metabolic profile changes were detected.

The data presented in this study, demonstrating the absence of adverse reactions in subjects actively ingesting *S. salivarius* K12, combined with the results of analysis of the biochemical, antibiogram, and virulence gene profiles of this bacterium, indicate that it has very low pathogenic potential and is unlikely to cause disease in healthy humans.

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