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Antimicrobial activity of *Streptococcus salivarius* K12 on bacteria involved in oral malodour

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ABSTRACT

Objective: To investigate the antimicrobial activity of the bacteriocin-producing strain *Streptococcus salivarius* K12 against several bacteria involved in halitosis.
Design: The inhibitory activity of *S. salivarius* K12 against *Solobacterium moorei* CCUG39336, four clinical *S. moorei* isolates, *Atopobium parvulum* ATCC33793 and *Eubacterium sulci* ATCC35585 was examined by a deferred antagonism test. *Eubacterium saburreum* ATCC33271 and *Parvimonas micra* ATCC33270, which have been tested in previous studies, served as positive controls, and the Gram-negative strain *Bacteroides fragilis* ZIB2800 served as a negative control. Additionally, the occurrence of resistance in *S. moorei* CCUG39336 to *S. salivarius* K12 was analysed by either direct plating or by passage of *S. moorei* CCUG39336 on chloroform-inactived *S. salivarius* K12-containing agar plates.
Results: *S. salivarius* K12 suppressed the growth of all Gram-positive bacteria tested, but the extent to which the bacteria were inhibited varied. *E. sulci* ATCC35585 was the most sensitive strain, while all five *S. moorei* isolates were inhibited to a lesser extent. Natural resistance seems to be very low in *S. moorei* CCUG39336, and there was only a slight decrease in sensitivity after exposure to *S. salivarius* K12 over 10 passages.
Conclusion: Our studies demonstrate that *S. salivarius* K12 has antimicrobial activity against bacteria involved in halitosis. This strain might be an interesting and valuable candidate for the development of an antimicrobial therapy for halitosis.
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1. Introduction

Oral malodour, also called halitosis, afflicts a significant proportion of the adult population and is of common interest due to its compromising influence in social and working environments. Most halitosis oral malodour compounds are by-products of the metabolism of certain species of oral bacteria, mainly those on the dorsum of the tongue.[1,2] These compounds consist of VSC (volatile sulphur compounds), valeric acid, butyric acid and putrescine.[2] A diverse group of Gram-negative and Gram-positive bacteria has been found to contribute to the problem. By contrast, certain bacterial species that predominate in the mouths of "healthy" subjects are noticeably absent in subjects with halitosis.[3]

Current treatments focus on the use of chemical or physical antibacterial regimens to reduce the numbers of these bacteria. The treatments typically provide only short-term relief because

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the offensive bacteria quickly recover after treatment is stopped.[4]

The use of probiotics has long been popular in the food industry. The World Health Organisation defines probiotics as a 'live organism which when administered in adequate amounts confers a health benefit on the host'. Their use in clinical practice has previously been discussed.[5] One potential and clinically important use of probiotics is in the prevention of dental caries.[6–10]

Preventing the re-growth of odour-causing organisms through the pre-emptive colonisation of the oral cavity with non-odorous, commensal microorganism may be a reasonable alternative to chemical or physical antibacterial regimens. Given that the dorsum of the tongue is the origin of most halitosis problems, a candidate probiotic to counter this condition should be able to persist in this particular ecosystem. The production of anti-competitor molecules such as bacteriocins also appears to confer an ecological advantage to some bacteria. A probiotic strain that efficiently colonises the tongue surface and does not produce odours metabolic by-products would be highly advantageous.

Streptococcus salivarius is known to be a pioneer coloniser of oral surfaces and is found predominant in 'healthy' humans not affected by halitosis.[3] BLIS K12 Throat Guard lozenges (BLIS Technologies, Centre for Innovation, Dunedin, New Zealand) contain *S. salivarius* K12, which has been shown to help maintain throat health by supporting the defence against undesirable bacteria.[11] The bacterium is not genetically modified or engineered, and the product is available in three flavours (vanilla, strawberry and peppermint). The particular strain used produces two natural antibacterial peptides, salivaricin A2[12,13] and salivaricin B,[14] which are lantibiotic-type bacteriocins. In deferred antagonism studies, *S. salivarius* K12 inhibited the Gram-positive bacteria *Streptococcus anginosis* T29, *Eubacterium saburreum* and *Micromonas micros*, which are implicated in halitosis, and significantly inhibited black-pigmented colony types present in saliva samples.[4]

Based on these investigations and other promising results, *S. salivarius* K12 has an excellent potential for use as a probiotic targeting halitosis producing bacteria.

The aim of this study was to evaluate the extent of the inhibitory spectrum of *S. salivarius* K12 against three additional bacterial species recently found to be implicated in halitosis and to investigate the development of bacterial resistance against *S. salivarius* K12.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacteriocin-producing strain *S. salivarius* K12 and the nonproducer *S. salivarius* MU, were kindly provided by Prof. J. Tagg (Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand).[4]

The indicator strains used in this study included the following: *E. saburreum* ATCC 33271; *Parvimonas micra* (previously known as *Micromonas micros* or *Peptostreptococcus micros*) ATCC 33270, which served as a positive control[4] and *Bacteroides fragilis* ZIB 2800 (School of Dental Medicine, University of Basel, Switzerland), which served as a negative control. The test strains included *Atopobium parvulum* ATCC 33793, *Eubacterium sulci* ATCC 35585, *Solobacterium moorei* CCUG 39336 and four clinical *S. moorei* isolates, CH1#23, CH3A#109A, CH3#63 and CH8#20,[15] which had, to date, not yet been tested for susceptibility against *S. salivarius* K12 *in vitro*.

All bacteria were grown on Columbia agar (Columbia Agar Base [BBL Becton Dickinson, Allschwil, Switzerland]) supplemented with 4 mg/l hemin (Fluka, Buchs, Switzerland), 1 mg/l menadione (VWR International, Dietikon, Switzerland) and 50 ml/l human blood (Blutspendezentrum, Basel, Switzerland) under anaerobic conditions (Oxoid AnaeroGen Compact, Oxoid, Pratteln, Switzerland) at 37 °C for 2–4 days.

2.2. Antimicrobial activity of *S. salivarius* K12

Inhibitory activities of *S. salivarius* K12 and the salivaricin non-producer *S. salivarius* MU were analysed using a modified deferred antagonism test.[16] Sterile blotting paper (Inapa Schweiz AG, Regensdorf, Switzerland) was cut to the size of 9 cm × 1 cm and carefully immersed in a *S. salivarius* culture with a density of 4-5 McFarland standard. After removing excess fluid, the blotting paper was placed in the middle of a plate of Columbia agar containing 5% human blood and 0.1% calcium carbonate ($CaCO_3$) (E. Merck, Darmstadt) left in place for 2 s and then removed. The plates were incubated at 37 °C under anaerobic conditions for 24 h. After incubation, the growth was removed with a sterile cotton swab. To kill any residual bacterial cells on the mediums surface, the plate was exposed to chloroform (E. Merck, Darmstadt) vapours for 30 min at room temperature. The plate was then aired for 30 min.

Several colonies of each indicator strain grown on Columbia blood agar-calcium carbonate medium were suspended in 3 ml Todd-Hewitt broth and streaked at right angles to the original *S. salivarius* culture zone with a sterile cotton swab. The plates were incubated under anaerobic conditions at 37 °C for at least 48 h, and the extent of inhibition was recorded in mm (the distance between the original producer line and the inhibition line of indicator strains). Each test was performed at least three times.

2.3. Test for resistance of *S. moorei* CCUG 39336 against *S. salivarius* K12

S. salivarius K12 or *S. salivarius* MU cells were each suspended in 3 ml Todd Hewitt broth and swabbed onto Columbia blood agar-calcium carbonate medium. Afterwards, the plates were incubated at 37 °C under anaerobic conditions for 24 h until confluent growth was observed. Bacterial cells were removed from the plates with sterile cotton swabs, and the agar surfaces exposed to chloroform vapour for 30 min and aired for another 30 min. Control plates without *S. salivarius* were also exposed to the same conditions.

To detect bacteriocin-resistant *S. moorei* isolates, several colonies of *S. moorei* CCUG 39336 were inoculated in 2 ml Todd-Hewitt broth. After incubation at 37 °C under anaerobic conditions for 24 h, 1 ml of this suspension was centrifuged at 10,000 rpm for 15 min at 15 °C and resuspended in 300 μl Todd-Hewitt broth. The exact cell density was determined by plating appropriate dilutions onto Columbia blood

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