Characterization of a broad-spectrum antimicrobial peptide from *Enterococcus mundtii* active against bacteria associated with middle ear infections

by

Hendriëtte Knoetze

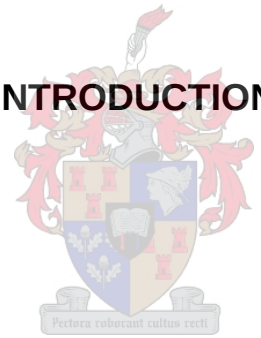
Thesis presented in partial fulfilment of the requirements for the degree of Master of Science at the University of Stellenbosch

Supervisor: Prof. L.M.T. Dicks

Co-supervisor: Dr. S.D. Todorov

December 2006

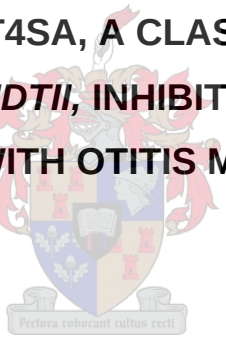
INTRODUCTION



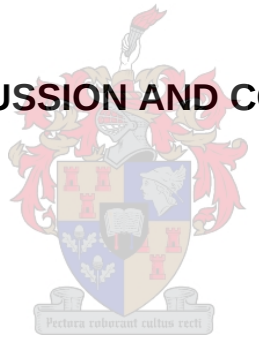
LITERATURE REVIEW

CHARATERIZATION OF BACTERIOCIN ST4SA, PRODUCED BY *ENTEROCOCCUS MUNDTII* ST4SA ISOLATED FROM SOYA BEANS

BACTERIOCIN ST4SA, A CLASS IIA PEPTIDE FROM *ENTEROCOCCUS MUNDTII,* INHIBITS BACTERIA ASSOCIATED WITH OTITIS MEDIA



FINAL DISCUSSION AND CONCLUSION



DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any other university for a degree.

Signature: _____ Date: _____

SUMMARY

Strain ST4SA, isolated from soya beans, was identified as *Enterococcus mundtii*. BacST4SA, a bacteriocin produced by strain ST4SA inhibited the growth of *Acinetobacter baumannii*, *Bacillus cereus, Clostridium tyrobutyricum, Enterococcus faecalis, Enterococcus faecium, Lactobacillus sakei, Propionibacterium* spp.*, Streptococcus caprinus, Pediococcus* sp., *Listeria monocytogenes, Staphylococcus aureus*, *Streptococcus pneumoniae*, and unidentified middle ear isolates A, BW, DW, F, G, and H. BacST4SA was active against *Pseudomonas aeruginosa* G, BG, I, J, B and E, although variable degrees of resistance were observed for some strains.

BacST4SA is positively charged, hydrophobic, contains the YGNGV sequence in the N-terminal, a double-glycine processing site and a disulphide bridge, all of which is typical of a class IIa bacteriocin. The operon, which contains a structural-, ATP-dependent transporter- and immunity gene, is located on a 50-kb plasmid. The 58-amino acid prepeptide is homologous to mundticin KS, mundticin AT06 and bacteriocin QU 2, and differs from enterocin CRL35 by only two amino acids. The 674-amino acid ATP-dependent transporter, consisting of a peptidase C39B domain, an ABC-transporter and an ABC-DLP family domain, displayed 98.9% homology to mundticin KS and 99.25% to enterocin CRL35. The 98-amino acid immunity gene of bacST4SA is completely homologous to enterocin CRL35 and 96.9% to mundticin KS.

BacST4SA is 3.950 kDa in size, based on electron spray mass spectrometry. The peptide was isolated from the cell-free supernatant, precipitated with 80% saturated ammonium sulphate, dialysed and freeze-dried to 1 638 400 AU (arbitrary units) per ml. No change in antimicrobial activity was recorded when bacST4SA was incubated in buffer ranging from pH 2 to 12, heated to 100 °C for 90 min and 121 °C for 20 min, and when incubated in the presence of Tween 20, Tween 80, Triton X-100, SDS, urea, EDTA, middle ear fluid and blood.

Optimal levels of bacST4SA production (51 200 AU/ml) was recorded after 14 h of growth in MRS broth at 30°C. Maximum production (102 400 AU/ml) was recorded in modified MRS media supplemented with tryptone, yeast extract, a combination of tryptone and yeast extract, K_2HPO_4 (10.0 or 20.0 g/l), or with the addition of DL-6,8-thioictic acid, L-ascorbic acid, and thiamine, respectively.

BacST4SA is bactericidal towards *E. faecium* HKLHS and bacteriostatic towards *S. pneumoniae* 40 and middle ear isolates F, BW and H. The peptide adsorbed maximal (94%) to *S. pneumoniae* 40, *P. aeruginosa* 25 and *E. faecium* HKLHS. BacST4SA forms pores in the cytoplasmic membrane of sensitive cells, leading to dissipation of the cell membrane and leakage of cytoplasmic material.

BacST4SA was compared with various other antimicrobial treatment agents, and revealed similar to a higher activity towards a number of these agents. BacST4SA revealed a similar level of activity against *E. faecium* HKLHS and middle ear pathogens *P. aeruginosa* J and *S. pneumoniae* 27 when compared with tetracycline (30µg). However, bacST4SA revealed much higher activity when compared to nasal sprays, aminoglycosides, cephalosporins, fluoroquinolones, lincosamides, macrolides, nitroimidazole, penicillin, quinolones, sulfonamides, chloramphenicol, furanzolidone, fusidic acid, rifampicin, trimethoprim, trimethoprim-sulfamethoxazole and vancomycin when tested *in vitro*.

OPSOMMING

Stam ST4SA, geïsoleer uit sojabone, is as *Enterococcus mundtii* geidentifiseer. BacST4SA, ‘n bakteriosien geproduseer deur stam ST4SA het die groei van *Acinetobacter baumannii*, *Bacillus cereus, Clostridium tyrobutyricum, Enterococcus faecalis, Enterococcus faecium, Lactobacillus sakei, Propionibacterium* spp.*, Streptococcus caprinus, Pediococcus* sp., *Listeria monocytogenes, Staphylococcus aureus*, *Streptococcus pneumoniae* en ongeïdentifiseerde middeloor isolate A, BW, DW, F, G, en H geinhibeer. BacST4SA is aktief teen *Pseudomonas aeruginosa* stamme G, BG, I, J, B en E, alhoewel effense weerstand soms waargeneem is.

BacST4SA het ‘n netto positiewe lading, is hidrofobies, bevat die YGNGV-volgorde in die N-terminaal, ‘n dubbel-glisien prosesserings setel en ‘n disulfied brug, kenmerkend van klas IIa bakteriosiene. Die operon, wat bestaan uit ‘n strukturele geen, ‘n ATP-afhanklike transport sisteem geen en ‘n immuniteits-geen, is op ‘n 50 kb plasmied gelokaliseer. Die voorloper peptied (58 aminosure lank), is homoloog aan mundticin KS, mundticin AT06 en bakteriosien QU 2 en verskil van enterocin CRL35 met slegs twee aminosure. Die ATP-afhanklike transporter (674 aminosure lank) bestaan uit ‘n peptidase C39B domein, ‘n ABC-transporter en ‘n ABC-DLP tipe domein en is 98.9% homoloog aan mundticin KS and 99.25% aan enterocin CRL35. Die immuniteits-geen (98 aminosure lank) van bacST4SA is ten volle homoloog aan enterocin CRL35 en 96.9% homoloog aan mundticin KS.

BacST4SA is 3.950 kDa groot, gebaseer op elektrosproei-massa spektrometrie. Die peptied is uit selvrye supernatant geïsoleer, met 80% versadigde ammonium sulfaat gepresipiteer, gedialiseer en gevriesdroog tot ’n finale konsentrasie van 1 638 400 AE (arbitrêre eenhede) per ml. Geen verandering in antimikrobiese aktiwiteit is waargeneem tydens inkubasie van bacST4SA in buffer van pH 2 tot 12, tydens verhitting (100 °C vir 90 min en 121 °C vir 20 min) en tydens inkubasie in die teenwoordigheid van Tween 20, Tween 80, Triton X-100, SDS, ureum, EDTA, middeloor vloeistof en bloed.

Optimale vlakke van bacST4SA produksie (51 200 AE/ml) is na 14 h groei in MRS media by 30°C waargeneem. Maksimale vlakke van die peptied (102 400 AE/ml) is geproduseer in gemodifiseerde MRS medium, aangevul met triptoon, gisekstrak, ‘n kombinasie van triptoon en gisekstrak, K_2HPO_4 (10.0 of 20.0 g/l), of met byvoeging van DL-6,8-thioktiensuur, L-askorbiensuur, en tiamien onderskeidelik.

BacST4SA is bakteriosidies teenoor *E. faecium* HKLHS en bakteristaties teenoor *S. pneumoniae* 40 en middeloor isolate F, BW en H. Die peptied adsorbeer optimaal (94%) aan *S. pneumoniae* 40, *P. aeruginosa* 25 en *E. faecium* HKLHS. BacST4SA vorm porieë in die selmembraan van sensitiewe selle en lei tot vernietiging van die selmembraan en lekkasie van die sitoplasma inhoud.

In vergelykende studies het bacST4SA ‘n soortgelyke en selfs hoër antimikrobiese aktiwiteit teenoor ‘n aantal bekende antimikrobiese middels getoon. Die aktiwiteit van bacST4SA is soortgelyk aan dié van tetrasiklien (30µg) in toetse teen *E. faecium* HKLHS en middeloor patogene *P. aeruginosa* J en *S. pneumoniae* 27. BacST4SA het egter in ’n *in vitro* vergelyking met neussproeie, aminoglisiedes, cephalosporiene, fluoroquinolone, lincosamides, makroliede, nitroimidazole, penisilien, quinolone, sulfonamide, chloramphenicol, furanzolidone, fusiensuur, rifampisien, trimethoprim, trimethoprim-sulfamethoxazool en vankomisien ‘n baie hoër aktiwiteit teen patogene getoon.

BIOGRAPHICAL SKETCH

Hendriëtte Knoetze was born on the 6th of May 1981 in Bellville. She matriculated from Strand High School in 1999 and thereafter enrolled at the University of Stellenbosch. In April 2003 she obtained her B.Sc. Molecular and cellular degree with Microbiology, Biochemistry and Genetics as majors. In December 2003 she obtained her B.Sc. (Hons.) in Microbiology.

PREFACE

The literature review includes an update on otitis media, the genus *Enterococcus*, and class II bacteriocins they produce.

The papers, “Characterization of bacteriocin ST4SA, produced by *Enterococcus mundtii* ST4SA isolated from soya beans”, and “Bacteriocin ST4SA, a class IIa peptide from *Enterococcus mundtii*, inhibits bacteria associated with otitis media”, has been written according to the style of Applied and Environmental Microbiology.

ACKNOWLEDGEMENTS

I sincerely want to thank:

Prof. L.M.T. Dicks, my study-leader, Department of Stellenbosch, Stellenbosch, for his guidance during my postgraduate studies and for giving me the opportunity to be part of this research group.

Dr. S.D. Todorov, Dr. C.A. van Reenen, Dr. S.M. Deane and Mr. L.J. van Zyl, Department of Microbiology, University of Stellenbosch, for their advice and patients.

My co-workers in the Department of Microbiology for their inputs and support.

The National Research Foundation (NRF), Cipla Medpro (Pty) Ltd and Shephard Medical (Pty) Ltd, South Africa, for funding this project.

My family and friends for their love, patients and support.

CONTENTS

INTRODUCTION

Otitis media (OM), an infection of the middle ear, affects more than 60% of children under the age of two (Rosenfeld *et al.,* 2004; Kouwen *et al.,* 2005). Most of the severe infections are caused by β-lactamase–producing pathogens, *Haemophilus influenzae* and *Moraxella catarrhalis*, and multi-drug resistant *Streptococcus pneumoniae* (McCracken, 2002; Klein, 1999; Segal *et al.,* 2005). Antibiotics used to treat these pathogens include high-dose amoxicillin, amoxicillin-clavulanate (augmentin), cefuroxime axetil and intramuscular ceftriaxone (Klein, 1999; McCracken, 2002; Segal *et al.,* 2005). The major problem encountered with antibiotic treatment of acute otitis media is the tremendous increase in the resistance to antibiotics (Klein, 1999; Rovers *et al.*, 2004; Segal *et al.,* 2005). If not treated, the disease may lead to complications in the aural cavity, intratemporal and intracranium areas, and may manifest in mastoiditis, meningitis and brain abscess (Eneli, 1998; Penido *et al.,* 2005).

In recent papers, ribosomal synthesized cationic peptides, known as bacteriocins, have been considered as an alternative to antibiotics (Delves-Broughton *et al.,* 1996; Ryan *et al.,* 1999; Cleveland *et al.,* 2001; Hancock and Patrzykat, 2002; Riley and Wertz, 2002). Class IIa bacteriocins have received particular interest in the food industry (Chen and Hoover, 2003; Moreno *et al.,* 2005) and as antiviral agent (Wachsman *et al.,* 1999; Wachsman *et al.,* 2003; Todorov *et al.,* 2005). Attempts to use bacteriocins in medicine have only been studied in a few cases, with some cationic peptides used with success in clinical trails (Giacometti *et al.,* 2000; Ingham *et al.,* 2003). An indolicin-like peptide, MBR-226 has successfully prevented catheter-related bloodstream infections in phase III clinical trails (Hancock and Patrzykat, 2002). Indolicin-like peptides have also been used for therapy of acute acne (in phase II clinical trails) and the eradication of methicillin-resistant *Staphylococcus aureus* in nares (Hancock and Patrzykat, 2002). Piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola,* is the first reported class IIa bacteriocin displaying *in vivo* antimicrobial activity against *Listeria monocytogenes.* However, piscicolin 126 could not eradicate intracellular bacteria (Ingham *et al.,* 2003). The lantibiotic nisin has shown to be active against *Staphylococcus aureus,* and vancomycin-resistant enterococci when tested *in vitro* (Brumfit *et al.,* 2002), and has been used to prevent colonization of chicken skins by *Salmonella typhimurium* (Natrajan and Sheldon, 2000). A two component bacteriocin, lacticin 3147, produced by *Lactococcus lactis*, and nisin have also shown to be effective in the control of surface-related infections, such as mastitis (Sears *et al.,* 1992; Ryan *et al.,* 1999). The use of cationic peptides as an antibiotic complement has also been observed. A study by Giacometti *et al.* (1999) reported increased *in vitro* activity of peptides cecropin P1,

indolicin, magainin II, nisin and renalexin combined with polymyxin E and clarithromycin, respectively, against *Pseudomonas aeruginosa*. In another combinational study, polycationic peptides ranalexin and polymycin E, doxycycline and clarytromycin showed synergism against clinical isolates of Gram-positive and Gram-negative aerobic bacteria (Giacometti *et al.,* 2000). Magainin II has shown to be synergistic with beta-lactam antibiotics (Giacometti *et al.,* 2000).

Enterococci have the ability to produce small peptides, known as enterocins (De Vuyst and Vandamme, 1994; Franz *et al.,* 1999). Enterocins are grouped into class I, class IIa, class IIb, class IIc and class III, with classes IIa and IIc the most abundant groups (Moreno *et al.,* 2005). These peptides are generally active towards closely related Gram-positive bacteria, including food-borne pathogens *Listeria*, with only a few enterocins active against Gram-negative species (Chen and Hoover, 2003; Franz and Holzapfel, 2004). Enterocin AS-48, a class IIc bacteriocin produced by *E. faecalis* S-48, is active towards a variety of Gram-negative bacteria (Ananou *et al.,* 2005). Enterocin 012, produced by *Enterococcus gallinarum,* inhibits the growth *P. aeruginosa* and *Escherichia coli* (Jennes *et al.,* 2000), whilst enterocin MR99 of *E. faecalis* inhibits *E. coli* (Sparo *et al.,* 2006). Enterocins have developed a great deal of interest as an approach to control food-borne diseases, to be used as starter cultures and biopreservative in various food products (De Vuyst and Vandamme, 1994; Franz *et al.,* 1999; Franz and Holzapfel, 2004; Moreno *et al.,* 2005). The use of enterocins in medicine is a completely new research field. However, the use of enterocin CRL35 as an antibiotic compliment on *Listeria* (Minahk *et al.,* 2004) and as an antiviral agent has been observed (Wachsman *et al.,* 1999; Wachsman *et al.,* 2003).

In this study, a lactic acid bacterium isolated from soya beans, was screened for the production of a bacteriocin inhibitory towards various lactic acid bacteria, food-borne- and middle ear pathogens. The strain was identified to species level and the genes encoding the peptide have been sequenced. The mode of activity was determined and the antimicrobial action compared with that of antibiotics currently used to treat otitis media.

REFERENCES

Ananou, S., A. Ga´lvez, M. Martý´nez-Bueno, M. Maqueda and E. Valdivia. 2005. Synergistic effect of enterocin AS-48 in combination with outer membrane permeabilizing treatments against *Escherichia coli* 0157:H7. J. Appl. Microbiol. 99, 1364-1372.

Brumfitt, W., M.R.J. Salton, and J.M.T. Hamilton-Miller. 2002. Nisin, alone and combined with peptidoglycan-moduling antibiotics: activity against methillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. J. Antimicrob. Chemother. 50, 731-734.

Chen, H. and D.G. Hoover. 2003. Bacteriocins and their food applications. Compr. Rev. Food. Science Food Safety 2, 82-100.

Cleveland, J., T.J. Montville, I.F. Nes, and M.L. Chikindas. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. 71, 1-20.

Delves-Broughton, J., P. Blackburn, R.J. Evans, and J. Hugenholtz. 1996. Applications of the bacteriocin, nisin. Antonie van Leeuwenhoek. 69, 193-202.

De Vuyst, L., and E.J. Vandamme. 1994. Antimicrobial potential of lactic acid bacteria. In: De Vuyst, L., and Vandamme, E.J. (Eds.). Bacteriocins of lactic acid bacteria: Microbiology, Genetics and Application. Blackie Academic and Professional, London. pp. 91-142.

Eneli, I.U. 1998. Otitis Media: An Update. Medical Update for Psychiatrist 3(5), 165-169.

Franz, C.M.A.P., and W.H. Holzapfel. 2004. The Genus Enterococcus: biotechnological and safety issues. In: Salminen, S. A. von Wright, A and Ouwenhand A. (Eds.). Lactic acid bacteria: microbiological and functional aspects, 3rd ed. Marcel Dekker Inc., New York. pp. 199-247.

Franz, C.M.A.P., W.H. Holzapfel, and M.E. Stiles. 1999. Enterococci at the crossroads of food safety? Int. J. Food Microbiol. 47, 1-24.

Giacometti, A., O. Cirioni, F. Barchiesi, M. Fortuna, and G. Scalise. 1999. *In vitro* activity of cationic peptides alone and in combination with clinical used antimicrobial agents against *Pseudomonas aeruginosa.* J. Antimicrob. Chemother. 44, 641-645.

Giacometti, A., O. Cirioni, M.S. Del Prete, A.M. Paggi, M.M. D'Errico, and G. Scalise. 2000. Combination studies between polycationic peptides and clinically used antibiotics against Gram-positive and Gram-negative bacteria. Peptides 21, 1155-1160.

Hancock, R.E., and A. Patrzykat. 2002. Clinical development of cationic antimicrobial peptides: from natural to novel antibiotics. Curr. Drug Targets Infect. Disord. 2, 79-83.

Ingham, A.B., M. Ford, R.J. Moore, and M. Tizard. 2003. The bacteriocin piscicolin 126 retains antilisterial activity *in vivo*. J. Antimicrob. Chemother. 51, 1365-1371.

Jennes, W., L.M.T. Dicks and D.J. Verwoerd. 2000. Enterocin 012, a bacteriocin produced by *Enterococcus gallinarum* isolated from the intestinal tract of ostrich. J. Appl. Microbiol. 88, 349-357.

Klein, J.O. 1999. Management of acute otitis media in an era of an increasing antibiotic resistance. Int. J. Pediatr. Otorhinolaryngol. 49(1), S15-7.

Kouwen, H., F.A.M. van Balen, and P.H. Dejonkere. 2005. Functional tube therapy for persistent otitis media with effusion in children: myth or evidence? Int. J. Pediatr. Otorhinolaryngol. 69, 943-951.

McCracken, G.H. 2002. Diagnosis and management of acute otitis media in the urgent care setting. Ann. Emerg. Med. 39, 413-421.

Minahk, C.J., F. Dupuy, and R.D. Moreno. 2004. Enhancement of antibiotic activity by sub-lethal concentrations of enterocin CRL35. J. Antimicrob. Chemother. 53, 240-246.

Moreno, M.R.F., P. Sarantinopoulos, E. Tsakalidou, and L. de Vuyst. 2005. The role and application of enterococci in food and health. Int. J. Food Microbiol. 106(1), 1-24.

Natrajan, N., and B.W. Sheldon. 2000. Efficacy of nisin-coated polymer films to inactivate *Salmonella typhimurium* on fresh broiler skin. J. Food Prot. 63, 1189-1196.

Penido, N.D.O., A. Borin, LC.N. Iha, V.M. Suguri, E. Onishi, Y. Fukuda, and O.L.M. Cruz. 2005. Intracranial complications of otitis media: 15 years of experience in 33 patients. Otolaryngol. Head Neck Surg. 132(1), 37-42.

Riley, M.A., and J.E. Wertz. 2002. Bacteriocins: evolution, ecology, and application. Annu. Rev. Microbiol. 56, 117-137.

Rosenfeld, R.M., L. Culpepper, K.J. Doyle, K.M. Grundfast, A. Hoberman, M.A. Kenna, A.S. Lieberthal, M. Mahoney, R.A. Wahl, C.R. Woods, Jr., and B.L. Yawn. 2004. Clinical practice guideline: otitis media with effusion. Otolaryngol. Head Neck Surg. 130, S95-S118.

Rovers, M.M., A.G.M. Schilder, G.A. Zielhuis, and R.M. Rosenfeld. 2004. Otitis media. Lancet 363, 465-473.

Ryan, M.P., J. Flynn, C. Hill, R.P. Ross, and W.J. Meaney. 1999. The natural food grade inhibitor, lacticin 3147, reduced the incidence of mastitis after experimental challenge with *Streptococcus dysgalactiae* in non-lactating dairy cows. J. Dairy Sci. 82, 2625-2631.

Sears, P.M., B.S. Smith, W.K. Steward, R.N. Gonzales, S.D. Rubino, S.A. Gusik, E.S. Kulisek, S.J. Projan, and P. Blackburn. 1992. Evaluation of a nisin-based germicidal formulation on teat skin of live cows. J. Dairy Sci. 75, 3185-3190.

Segal, N., E. Leibovitz, R. Dagan, and A. Leiberman. 2005. Acute otitis media-diagnosis and treatment in the era of antibiotic resistant bacteria: updated clinical practice guidelines. Int. J. Otorhinolaryngol. 69, 1311-1319.

Sparo, M.D., M.S. Castro, P.J. Andino, M.V. Lavigne, C. Ceriani, G.L. Gutie´ rrez, M.M. Ferna´ndez, M.C. De Marzi, E.L. Malchiodi and M.A. Manghi. 2006. Partial characterization of enterocin MR99 from a corn silage isolate of *Enterococcus faecalis*. J. Appl. Microbiol. 100, 123-134.

Todorov, D.T., M.B. Wachsman, H. Knoetze, M. Meincken, and L.M.T. Dicks. 2005. An antibacterial and antiviral peptide produced by *Enterococcus mundtii* ST4V isolated from soya beans. Int. J. Antimicrob. Agents 25, 508-513.

Wachsman, M.B., V. Castilla, A.P. De Ruiz Holgado, R.A. de Torres, F. Sesma and C.E. Coto. 2003. Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication *in vitro*. Antiviral Res. 58, 17-24.

Wachsman, M.B., M.E. Farias, E. Takeda, F. Sesma, A.P. De Ruiz Holgado, R.A. de Torres, and C.E. Coto. 1999. Antiviral activity of enterocin CRL against herpes virus. Int. J. Antimicrob. Agents 12, 293-299.

1. Middle ear infection, otitis media

1.1. Introduction

Otitis media (OM) is the accumulation of fluids in the middle ear, with or without symptoms of inflammation. The infection is caused by dysfunction or obstruction of the eustachian tube (Kouwen *et al.,* 2005). It is the most commonly diagnosed upper respiratory tract infection (URTI) in children under the age of 2, for which antibiotics are prescribed, and has a high morbidity and low mortality rate (McCaig and Hughes, 1995; Segal *et al.,* 2005).

The microbiology of OM differs, with *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* (NTHI), and *Moraxella catarrhalis* the most commonly isolated pathogens (Block, 1997; McCracken, 2002). The increasing emergence of penicillin-resistant *S. pneumoniae* and β-lactamase-producing strains, NTHI and *M. catarrhalis*, has become a major health issue, making the diagnosis and treatment of OM a priority (Segal *et al.,* 2005). Health care costs associated with treatment are substantial, especially in cases of unresponsive treatment as a result of incorrect diagnosis (McCracken, 2002). Otitis media is classified into three main types: (1) otitis media with effusion (OME), i.e. middle ear effusion without symptoms of inflammation; (2) acute otitis media (AOM), i.e. middle ear effusion with symptoms of acute inflammation; and (3) chronic suppurative otitis media (CSOM), i.e. chronic inflammation of the middle ear, as a result of untreated or prolonged AOM, associated with perforation or poor response to treatment (Cripps *et al.,* 2005). Some cases of OM may resolve spontaneously, while most cases require antibiotic treatment. Recognition of risk factors is crucial for clinical management (Tong *et al.,* 2006).

In the United States approximately 3.5 billion US dollars are spent annually on antibiotic treatment of AOM. Prevention of a small percentage of cases will have a major impact on health and economical resources (Segal *et al.,* 2005). To combat the persistent spread of OM, research on alternative treatment has developed considerably, especially on pneumococcal vaccines (Cripps *et al.,* 2005). Another extensive focus is the bacterial interference (BI) of natural nasopharyngeal flora and their production of antibacterial compounds against pathogens (Brook, 2005).

To prevent the increasing occurrence of antibiotic resistance, the American Academy of Pediatrics (AAP) and the American Academy of Family Physicians (AAFP) have developed guidelines for the diagnosis and management of OM (AAP and AAFP, Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004).

1.2. The human ear

1.2.1. Anatomy of the ear

The human ear is part of the peripheral nervous system (Vander *et al.,* 2001). It is divided into three main sections: the outer/external, the middle-, and the inner-ear (Fig. 1). The middle ear is a small, air-filled chamber consisting of a tympanic membrane (TM) and ossicles (malleus, incus, and stapes) joined together, serving as a mechanical sound transformer. The TM seals off the outer auditory canal from the middle ear chamber and prevents micro-organisms from entering the middle ear and causing infection. The middle ear is connected to the nasopharynx via the eustachian tube. The inner-ear is a helically-shaped bony structure and is the most complex part of the auditory system. It consists of the cochlea, the vestibule and semicircular canals. The cochlea is a small bony structure with sensory nerves that convert sound waves to impulses. The vestibule and semicircular canals play a role in balance (Vander *et al.,* 2001).

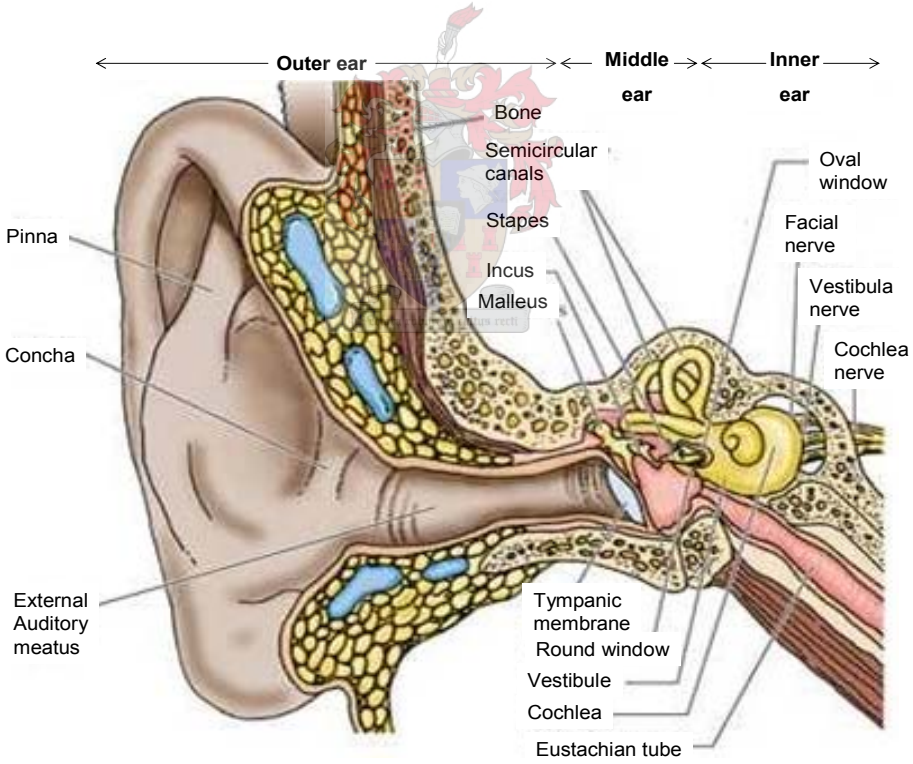


Fig. 1. Anatomy of the human ear (http://www.utdallas.edu/~tres/integ/sen5/sense_5.html).

1.2.2. The Eustachian tube and mucociliary system of the middle ear

The eustachian tube is a bony structure that starts at the anterior wall of the tympanic cavity and ends in the nasopharynx (Eneli, 1998). The nasopharyngeal passage remains

closed at rest and opens with swallowing, yawning, or forced inflation (de Ru and Grote, 2004).

The nasopharyngeal opening of the eustachian tube and anterior section of the middle ear are covered with ciliated columnar epithelial cells. The ciliary activity is sensitive to bacteria, allowing pathogens to attach and protect against infection. The eustachian tube consists of goblet cells, and two subepithelial mucous gland layers (the large dorso-caudal layer and a smaller ventro-cranial gland layer). Large mucus glands are situated in the lamina popria and adjacent wall of the nasopharynx orificium (Cayé-Thomasen and Tos, 2004). Mucus is normally transported across the middle ear mucosa, down the eustachian tube, enters the nasopharynx and is swallowed. The mucus consists of water, cells, cell debris, immunoglobins, lysozyme, lactoferrin, antimicrobial peptides, leukotrienes and cytokines, which protect the middle ear against invading pathogens (de Ru and Grote, 2004). The eustachian tube and mucosal system share several physiological functions (see Table 1).

Table 1: Physiological functions of the eustachian tube and mucociliary system with respect to the middle ear

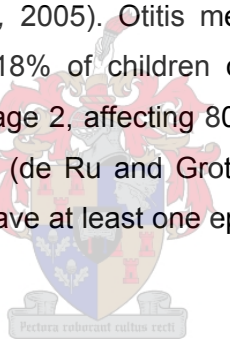
Eustachian tube function[a, b]	**Mucociliary system function** [c]
1. **Ventilation** of middle ear, equalising air pressure with atmospheric pressure caused by gas exchange between cavity and surrounding mucosa	1. Physical removal of bacteria, dust, viruses and allergens by ciliary **clearance**
2. **Drainage** of nasopharyngeal secretions and debris from the middle ear towards the nasopharynx with its mucociliary epithelium	2. Presence of a broad-spectrum of **antimicrobial agents** in the mucus
3. **Protection** from excessive nasopharyngeal secretions and fluctuations in the nasopharynx	3. Recruitment of phagocytotic cells and an **inflammatory response**

[a] Eneli, 1998; [b] Kouwen *et al.,* 2005; [c] de Ru and Grote, 2004

Normal functioning of the eustachian tube and mucociliary system are important for maintaining a healthy middle ear cavity (de Ru and Grote, 2004).

1.3. Epidemiology

Otitis media continues to be one of the most commonly diagnosed childhood infections (Rosenfeld and Bluestone, 1999). An estimated 2.2 million episodes per annum, at a total cost of 4.0 billion US dollars, affects more than 90% children in preschool and 60% in the first two years after birth (Rosenfeld *et al.,* 2004). Otitis media is the most common condition in children under two years of age and is most prevalent between six months and eighteen months of age (Eneli, 1998; Klein, 1989; Bamberger and Jackson, 2001). At least 60-65% of children will experience at least one episode of AOM within their first year, and approximately 17-30% will have suffered more than three episodes (Eneli, 1998; Fendrick *et al.*, 2001; McCracken, 2002; Segal *et al.,* 2005). Population-based studies in Finland and the USA have revealed an increase in AOM over the past 10 to 20 years. This is directly linked to an increase in the number of children attending day-care centers (Barden *et al.,* 1998; Gould, 1998). Children younger than three years of age are more prone to OM because of a narrow, shorter, less cartilaginous, and horizontal eustachian tube, making the functions less effective (Eneli, 1998; Kouwen *et al.,* 2005). Otitis media with effusion (OME) has been reported in 5-13%, 11-20% and 13-18% of children of ages one, three and five years, respectively, with the greatest risk at age 2, affecting 80% of pre-school children, and being the most common reason for surgery (de Ru and Grote, 2004; Kouwen *et al.,* 2005). It is estimated that nearly every child will have at least one episode of OME before 4 years of age (Zielhuis *et al.,* 1990).



Host-, extrinsic- and protective factors increase the risk of OM, and may play a role in persistent effusion and chronic disease. Host factors include age, craniofacial abnormalities (cleft palate), large adenoids, atopic history, recent viral URTI, recent sinusitis, eustachian tube dysfunction, immune deficiency or suppression disorders, and a family history of OM. Exposure to tobacco smoke and attendance of day-care programmes are considered extrinsic factors. Breast feeding for at least three months confers protection in the first year (Bamberger and Jackson, 2001; Eneli, 1998; Fendrick *et al.*, 2001; Segal *et al.,* 2005). The season of birth does not predict an episode of AOM or an early onset of AOM. However, children born during summer and autumn tend to attract AOM more easily and are at higher risk. The highest incidence of OM is usually during spring, autumn and winter, and lowest in summer (Homoe *et al.,* 2005). Winter months are always peak season for respiratory viruses (Bamberger and Jackson, 2001). A higher incidence of AOM has been recorded amongst males (Eneli, 1998). The reason for this is unknown.

The geographic distribution of OM pathogens, especially antibiotic-resistant pneumococci, follows a certain pattern. This is not fully understood, however it may be a result of social differences or prescription practices in different parts of the world (Reinert, 2004). In the 1940s penicillin was introduced as an alternative for the treatment of infectious diseases (Jacobs, 2004). Antibiotic resistance, first observed in 1967, has increased rapidly (Hansman and Bullen, 1967; Jacobs, 2004). The first report of multi-drug-resistant pneumococci (MDRP), *S. pneumoniae,* was published in the 1980s (Klein, 1999). This, and β-lactamase-producing strains of *H. influenzae* and *M. catarrhalis,* are considered major health threats.

In South Africa, MDRP was first recognised in 1977 and has increased since then. National surveys covering 1979-1986, 1979-1990 and 1991-1998, support the increase in multi-drug resistance (Klugman and Koornhof, 1988; Koornhof *et al.,* 1992; Witte, 1999; Huebner *et al.,* 2000). Although an increase in the number of resistant strains has been reported, the proportion of strains with high-level resistance has remained the same. A survey conducted from 1999 to 2002 revealed a slight decrease (1.8%) in the number of penicillin-resistant strains. Multi-drug-resistant phenotypes accounted for less than 10% of all isolates in South Africa, Brazil and Europe (Jones *et al.,* 2003). Penicillin resistance has increased markedly for β-lactamase-producing strains over the past decade. In a 1997 AOM case study, approximately 30% of the isolated penicillin-resistant strains *H. influenzae* and all strains of *M. catarrhalis* were β-lactamase positive (Segal *et al.,* 2005).

Resistance of *S. pneumoniae* to penicillin, other β-lactam antibiotics, macrolides and trimethoprim-sulfamethoxazole is of concern throughout the USA (McCracken, 2002). Currently, 14% of *S. pneumoniae* strains are resistant to three or more classes of antibiotics (Whitney *et al.,* 2000). Forty percent of penicillin-resistant *S. pneumonia* are resistant to trimethoprim-sulfamethoxazole and 30% to macrolides (Dowell *et al.,* 1999; Jacobs *et al.* 1999). Increased resistance to tetracycline, chloramphenicol and co-trimoxazole is an additional point of concern (Appelbaum, 1992; Appelbaum, 1995).

Sporadic cases of penicillin-resistant pneumococci have been reported from various parts of the world since about 1964. During the 1990s, penicillin-resistant *S. pneumoniae* (PRSP) increased in the USA, with incidences of 40% among children in day-care centres and 17% among children diagnosed with AOM (Segal *et al.,* 2005). Serotypes 19A, 14 and 23F developed high-level penicillin resistance and multiple resistances to other antimicrobials. The original strain representing serotype 23F originated in Spain, but clones thereof were subsequently isolated in Portugal, France, Croatia, USA, Mexico, and recently also in South

Korea and South Africa (Munoz *et al.,* 1991; Sibold *et al.,* 1992). Resistance to antibiotics amongst clones of serotype 23F differs (Stein *et al.,* 2003). One explanation for the latter is mutational changes in penicillin-binding proteins (PBPs) (Segal *et al.,* 2005). In an Israeli study, 50-70% of strains recovered from children with AOM were PRSP, with 10-30% of these strains resistant to macrolides, 50% to trimethoprim/sulphamethoxazole and 17% to a combination of antibiotics (Leibovitz and Dagan, 2000; Leibovitz and Dagan, 2001a; Leibovitz and Dagan, 2001b).

1.4. Pathogenesis and microbial etiology

Otitis media is caused by bacteria and viruses in other upper respiratory tract infections, normally the nasopharynx. Entrance to the middle ear is facilitated by dysfunctional mucociliary and an abnormal, poorly ventilated eustachian tube (Kouwen *et al.,* 2005). The nasopharynx acts as a bacterial reservoir for AOM (Kaieda *et al.,* 2005). Obstruction of the eustachian tube causes accumulation of fluids by surrounding epithelial cells and inflammation of the mucoperiosteal lining of the middle ear, resulting in OME and AOM, respectively (Bamberger and Jackson, 2001; de Ru and Grote, 2004). Infection is aggravated by viral URTI, allergies, smoke, anatomical abnormalities and swollen adenoids (Eneli, 1998; Kouwen *et al.,* 2005; Tong *et al.,* 2006). Smoke decreases ciliary beat frequency, which leads to clogging of the respiratory tract with mucous and adherence of pathogens to pharyngeal cells (Fainstein and Musher, 1979; Vastag *et al.,* 1986). Allergies cause dysfunction of the eustachian tube as a result of upper respiratory swelling, resulting in impaired mucociliary activity and increased colonisation of microbial cells (Kvaerner *et al.,* 1996; Fireman, 1997; Hastie *et al.,* 1997; Tanaka *et al.,* 1998).

S. pneumoniae consists of various virulent determinants, rendering the organism more pathogenic. The virulent determinants include surface protein adhesion and secretory IgA protease involved in colonisation to epithelial cells and capsules (Alonsodevelasco *et al.,* 1995). Based on the antigenic properties of the capsules, more than 90 serotypes have been classified. In AOM a number of serotypes may be present. However, strains of serological groups 1, 3, 4, 6, 7, 9, 14, 15, 18, 19 and 23 account for up to 85% of infections in children (Bamberger and Jackson, 2001; Sokos, 2005). *S. pneumoniae* causes severe AOM infection for up to 10 days, followed by an increase in the eustachian tube gland volume and goblet cell density 6 and 3 months after AOM. *S. pneumoniae* is known for its ability to induce change in bone tissue structures (osteoresorption and osteoneogenesis). This may be due to

a decrease in the ventilation spaces, causing predisposing morbidity (Cayé-Thomasen and Tos, 2004).

Non-typeable *H. influenzae* is responsible for 23-40% of bacterial cases of AOM, causing a milder but more prolonged course. *H. influenzae* biotypes 2 and 3 are predominant in OM, with only 10% type b (Eneli, 1998; Bamberger and Jackson, 2001; McCracken, 2002). Type b *H. influenzae* causes a short but severe AOM, while *M. catarrhalis* is responsible for 10-15% of cases, and induces only a mild but purulent course of infection. Non-typeable *H. influenzae* increases more severe or protracted histopathological changes in the middle ear, causing a dramatic increase in the eustachian tube gland volume and goblet cell density, with the formation of polyp and fibrous adhesion, leading to mucosal scarring. The increase usually persists for 6 months after AOM, whereas the increase in eustachian tube gland volume occurs after 3 months (Cayé-Thomasen and Tos, 2004). *M. catarrhalis* induces the mildest and slightest change, with an increase in goblet cell density for only a short period of infection, and with no increase in the eustachian tube gland volume. Differences in response are due to different effects of bacterial capsules or exotoxins on the immune system, causing different patterns of host response (Cayé-Thomasen and Tos, 2004).

Other pathogens less frequently isolated include *Staphylococcus aureus* (3%), group A streptococci (1%), *Streptococcus pyogenes* (1-5%) and Gram-negative bacilli (Eneli, 1998; McCracken, 2002; Segal *et al.,* 2005). Gram-negative enteric pathogens, *S. aureus* and group B beta-hemolytic streptococci are found in neonates but rarely encountered in older infants, children and adults (Turner *et al.,* 2002). *Pseudomonas aeruginosa* has been found in 67% of cases, with less than 1% of cases caused by *Mycobacterium tuberculosis* (Bamberger and Jackson, 2001).

Respiratory viruses are important co-pathogens and may contribute to the etiology and pathogenesis of AOM by causing inflammation of the mucosa and blockage of the eustachian tube. This impairs the host's immunity and increases the risk of attracting pathogens (Buchman and Brinson, 2003; Heikkinen and Chonmaitree, 2003; Kleemola *et al.,* 2006). Viral URTI causes eustachian tube failure and leads to build-up of pressure in the middle ear (de Ru and Grote, 2004). Kleemola *et al.* (2006) reported no distinct species-specific association between viral and bacterial infections. Viruses may precede clinical ear symptoms with or without bacteria (Kleemola *et al.,* 2006). Depending on the methods of detection, viruses account for up to 20-41% of infections, while 65% are caused by bacteria (Eneli, 1998; Heikkinen *et al.,* 1999; Nokso-Koivisto *et al.,* 2004; Segal *et al.,* 2005). Respiratory syncytial virus (RSV) is the most commonly isolated. Others include rhinovirus,

parainfluenzae, influenza A, enterovirus and adenovirus (Eneli, 1998; Bamberger and Jackson, 2001; Segal *et al.,* 2005).

1.5. Diagnosis

Upper respiratory tract infections have an enormous impact on the economy of communities and health care systems worldwide. Thirty to fifty percent of infants and children are diagnosed incorrectly and contributes to the misuse of medication and increase in antibiotic resistance (Lyon *et al.,* 1998; McCracken, 2002; Blomgren and Pitkäranta, 2005). Diagnostic procedures have to be cost-effective, quick and easy, with high sensitivity, specificity and accuracy for the detection of fluids in the middle ear (Blomgren and Pitkäranta, 2003; Blomgren and Pitkäranta, 2005; Segal *et al.,* 2005).

Accurate diagnosis of various types of OM are based on several symptoms of inflammation, including the colour, position, movement and bulging of the tympanic membrane, the presence or absence of effusions in the middle ear cavity, and the abrupt onset of OM (Blomgren and Pitkäranta, 2003; Blomgren and Pitkäranta, 2005; Segal *et al.,* 2005). Pneumatic otoscopy is based on direct visualisation of TM anatomy and colour, degree of mobility and the presence of effusions with over 90% sensitivity, and nearly 80% specificity (Eneli, 1998; Bamberger and Jackson, 2001; McCracken, 2002; Blomgren and Pitkäranta, 2003; Blomgren and Pitkäranta, 2005). If the pneumoscope is inconclusive, tympanometry and acoustic reflextometry are used, providing more evidence about the presence of middle ear effusion (McCracken, 2002). Tympanometry is the indirect measurement of the TM compliances and an estimation of middle ear pressure, with 90% efficacy. Sound waves are introduced to the TM and measurements are traced out graphically, indicating normal or poor compliance, flaccid or stiff TM, and eustachian tube dysfunction without middle ear effusion (Eneli, 1998). Prior to the use of tympanometry, symptoms have to be present, and results have to be interpreted in conjunction with the patient's history and clinical examination (Blomgren and Pitkäranta, 2003; Blomgren and Pitkäranta, 2005).

Diagnosis of AOM is based on the history of acute onset and the presence of inflammation (Segal *et al.,* 2005). A majority of symptoms may refer to the presence of inflammation, including fever, otalgia, otorhea, irritability, excessive crying, vomiting, diarrhoea, anorexia, URT symptoms, restlessness, poor feeding, ear tugging and emesis (Eneli, 1998; de Ru and Grote, 2004; Montgomery, 2005; Segal *et al.,* 2005). Pain is

generally a definite association with inflammation. The TMs of AOM patients are generally hyperaemic, bulging, cloudy, opague, thickened, immobile, and red to pale yellow with greyish effusion (Eneli, 1998; AAP and AAFP, Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004; Segal *et al.,* 2005). Otitis media with effusion cannot be diagnosed symptomatically and is detected using pneumoscopy, supplemented with tympanometry or acoustic reflectometry. Behavioural symptoms such as hearing loss and tinnitus might be present, with discomfort and little pain (Eneli, 1998; Segal *et al.,* 2005). The TM is non-perforated, bulged and full, with a decrease in mobility (Cripps *et al.,* 2005; Montgomery, 2005).

1.6. Management in an era of antibiotic resistance

1.6.1. Treatment

In the past AOM was easy to treat, but due to increasing antibiotic resistance OM has become difficult to manage (Pichichero, 2000a). Given the high prevalence of drug-resistant *S. pneumoniae* (DRSP) and β-lactamase-producing pathogens, the effectiveness in treatment is very important. Treatment strategies include medication for pain, antibiotic medication with or without steroids, myringotomy with or without insertion of ventilation tubes, adenoidectomy, and spontaneous resolution based on age, health, medical history and extent of disease (Paap, 1996; AAP and AAFP, Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004; Rovers *et al.,* 2004). The AAP, the AAFP, and the American Academy of Otolaryngology-Head and Neck Surgery have developed guidelines for the management of OME and AOM to combat the increase in the high occurrence of antibiotic-resistant pathogens (AAP and AAFP, Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004; AAFP, American Academy of Otolaryngology-Head and Neck Surgery, AAP; Subcommittee on Otitis Media with effusion, 2004).

The first and very critical component is the management of pain within 24 to 36 hours after diagnosis. Oral treatment includes the use of acetaminophen (15 mg/kg per dose every 4 to 6 hours) and ibuprofen (10 mg/kg per dose every 6 hours) as adequate analgesia (AAP and AAFP, Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004).

Various studies reported cases of AOM managed by spontaneous resolution (withholding antibiotics), which resulted in 85% eradication in some cases (Bamberger and

Jackson, 2001). Resolution of AOM is generally 60% at one month, 80% at two months and 90% at three months (Eneli, 1998). By decreasing the use of antibiotics with 50%, a 50% decrease in antibiotic-resistant bacteria was observed (Seppälä *et al*., 1997; Spiro *et al.*, 2005). Spontaneous resolution of AOM occurred in 90%, 70% and 20% of cases caused by *M. catarrhalis,* NTHI and *S. pneumoniae*, respectively (Bamberger and Jackson, 2001; Fendrick *et al.*, 2001; McCracken, 2002). The low eradication percentage for *S. pneumoniae* may be of major concern because *S. pneumoniae*, the predominant agent causing AOM, is associated with complications such as meningitis and pneumoniae and often requires hospitalisation. During the last five years, 20-30% of *S. pneumoniae* strains developed resistance to penicillin (Bamberger and Jackson, 2001; Fendrick *et al.,* 2001).

According to the AAP and AAFP Clinical Practice Guidelines (2004) spontaneous eradication should only be considered for healthy children six months to two years of age, with an uncomplicated or an uncertain diagnosis of AOM. Complications include anatomical abnormalities such as cleft palate, genetic conditions such as Down syndrome, immunodeficiencies, and cochlear implants. Children older than two years of age may use spontaneous eradication even if diagnosis is uncertain, or certain but not severe. Children younger than three to six months have to use antibiotics even if diagnosis is uncertain. Spontaneous resolution has to be observed for a period of 48 to 72 hours (AAP and AAFP Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004).

Resolution occurs over a period of time, while antibiotics are used for eradication of pathogens from the site of infection over a shorter period of time and to avoid possible local complications (Fendricks *et al.,* 2001; AAP and AAFP Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004). Therefore, withholding antibiotics may not be the prudent choice in all cases. More research has to be done to evaluate this method and its possible link to resistance (Fendricks *et al*., 2001). To use an antibiotic, a significantly better outcome has to be achieved with the drug than achieved by spontaneous eradication (Segal *et al.,* 2005).

Antibiotic treatment is based on eradication of pathogens and penetration of sufficient levels of the antibiotic into the middle ear (Dagan and Leibovitz, 2002). Various factors have to be considered when choosing an appropriate antibiotic. These include compliance and susceptibility. Antibiotic resistance patterns also have to be determined. Taste and palatability, dosing interval, proven clinical efficacy, history of drug allergies, costs, and previous response to different antibiotic regimens must also be taken into account (Eneli, 1998). Currently, no single drug eradicates all pathogens involved in AOM (Segal *et al.,*

2005). Resistance patterns vary in different communities and geographical locations and influence antibiotic prescription (Eneli, 1998). It is important to determine the minimum inhibitory concentration (MIC) against the causative pathogen (Dagan and Leibovitz, 2002; Kaieda *et al.,* 2005). Most antibiotics are β-lactams, acting in a time-dependent killing mechanism. The major determinant of efficacy is the time the drug concentration at site of infection exceeds the MIC for pathogens. An effective dose regimen in middle ear fluid concentration exceeds the MIC values for a pathogen by at least 40-50% of the dosing concentration (Segal *et al.,* 2005).

High-dose amoxicillin (80-90 mg/kg per day) for 10 days is used as first-line therapy in cases with a high prevalence of penicillin-resistant pneumococci, yielding middle ear fluid levels of amoxicillin that exceeds the MIC of many resistant strains of *S. pneumoniae* (Bamberger and Jackson, 2001; AAP and AAFP, Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004; Segal *et al.,* 2005). Amoxicillin has the greatest *in vitro* inhibition against *S. pneumoniae,* an excellent pharmacokinetic profile, and is relatively inexpensive. However, amoxicillin is not effective against β-lactamase-producing strains *M. catarrhalis* (50-100%) and *H. influenzae* (40-60%) (Eneli, 1998; AAP and AAFP, Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004; Segal *et al.,* 2005). If patients do not respond to therapy within 48 to 72 hours, the antibiotic treatment has to be changed.

Second-line agents include amoxicillin/clavulanic acid, cefuroxime axetil, and intramuscular ceftriaxone. Amoxicillin/clavulanic acid or augmentin for 10 days (90 mg/kg per day of amoxicillin component, with 6.4 mg/kg per day of cluvanate divided into two dosages) is effective to eradicate β-lactamase-producing bacteria and is used in severe illnesses were moderate to severe otalgia or a fever of more than 39 °C are observed (Segal *et al.,* 2005). Cephalosporin and cefuroxime axetil are only used if severe allergies occur, displaying the greatest *in vitro* activity against PRSP and also against β-lactamase-producing bacteria. Cephalosporins and cefuroxime axetil are known for poor palatability (McCracken, 2002; Montgomery, 2005; Segal *et al.,* 2005). In cases of children vomiting or in a situation that precludes administration of oral antibiotics, ceftriaxone (50 mg/kg/day for 3 days) is used (Bamberger and Jackson, 2001; McCracken, 2002; AAP and AAFP, Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004; Segal *et al.,* 2005). Parenteral ceftriaxone (effective as a single-dose) is effective against all common pathogens, especially resistant streptococci, but is expensive and has a broad activity range. Parenteral ceftriaxone is only required for patients unable to use oral antibiotics (Eneli, 1998; Bamberger and Jackson, 2001).

Patients with non-type 1 allergic reaction to amoxicillin are prescribed cefdinir (14 mg/kg/day once daily), cepodoxime or cefuroxime (30 mg/kg/day), while those with type 1 hypersensitivity allergies are prescribed macrolides, including azithromycin (10 mg/kg/day on day 1, followed by 5 mg/kg/day for 4 days as a single dose daily), or clarithromycin (15 mg/kg/day bid) as alternative therapy (McCracken, 2002; Montgomery, 2005; Segal *et al.,* 2005). Trimethoprim/sulfamethoxazole and erythromycin-sulfisoxazole are only used in cases of severe penicillin allergies, due to their high prevalent resistance (Bamberger and Jackson, 2001; McCracken, 2002; AAP and AAFP, Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004; Segal *et al.,* 2005). Third-line agents include clindamycin (Eneli, 1998). Clindamycin (30-40 mg/kg/day in 3 separate doses) is recommended in cases of penicillin resistance. However, it is ineffective against Gram-negative bacteria and will not act against *H. influenzae* and *M. catarrhalis* (McCracken, 2002; Montgomery, 2005; Segal *et al.,* 2005).

With the use of antibiotics, side effects have to be considered. Trimethoprim/sulfamethoxazole has been associated with Steven-Johnson syndrome and had an increased failure rate of 79% and 46% in *S. pneumoniae* and *H. influenzae*, respectively, suggesting increasing resistance. Augmentin may cause a rash and diarrhoea (Bamberger and Jackson, 2001; Segal *et al.,* 2005). Infants and toddlers are recommended a 10-day oral antibiotic treatment, while older patients are recommended a five to seven-day treatment. If symptoms deteriorate within two days, tympanocentesis is required. Younger patients have to be re-evaluated for clinical resolution in two to three weeks from initiation therapy (Bamberger and Jackson, 2001).

Recently, new quinolones (gafifloxacin and levofloxacin) were studied as an alternative to treat acute otitis media. Gafifloxacin and levofloxacin are 100% efficient in the eradication for *H. influenzae*. Levofloxacin and gafifloxacin eradicated 84% and 94% of *S. pneumoniae*, respectively, after four to six days. The success rate of the treatment is 90-94%. None of these two antibiotics have yet been licensed for use in paediatric AOM (Segal *et al.,* 2005). Research is also conducted on the use of oxazolidinones, streptogramins and ketolides (Pichichero, 2000b).

Otitis media with effusion generally develops after AOM. In 50% of cases, effusion directly follows an episode of infection (de Ru and Grote, 2004). According to de Ru and Grote (2004), OME should not be treated. The authors argue that it is selflimiting, and more likely to be a well-balanced response, regulated by the host's innate and aquired immune

system to protect the body. Research has indicated the presence of several factors of the immune system in middle ear effusions induced by the middle ear mucosa. Several cytokines are produced to control acute inflammation (de Ru and Grote, 2004). Bacterial metabolites, peptidoglycan-polysaccharide (PG-PS) and endotoxin may also induce the production of cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-1β (Schousboe *et al.,* 2001; Kawano *et al.,* 2002). The latter cytokines induce neutrofil infiltration, increasing capillary permeability, and promote development of subepithelial edema in the middle ear resulting in effusion (Lee *et al.,* 2001). Tumor necrosis factor-α and IL-1β induce goblet cells, leading to increased secretion of mucin and OME. With additional stimulation of IL-8, mucin secretion from the goblet cells is prolonged and results in long-term progression of OM (Smirnova *et al.,* 2002).

In healthy children, first-line treatment for OME is spontanoues resolution by adjusting environmental factors responsible for causing obstruction of the eustachian tube. Guidelines for the management of OME, developed by the Guidelines and Protocols Advisory Committee (2004), are only recommended for healthy children over the age two months, without craniofacial abnormalities, immune deficiencies, AOM complications or other serious underlying diseases. Spontaneous resolution is recommended in children with no risks or complications. If OME does not resolve spontaneously, the history and risks of disease have to be examined (de Ru and Grote, 2004). Antibiotics may hasten resolution of OME in only 14% of cases, making antibiotic treatment generally ineffective (Guidelines and Protocols Advisory Committee, 2004). Rosenfeld and Kay (2003) demonstrated spontaneous resolution in 20-56% of cases after three months. If effusions are still present after three months, surgery has to be considered (AAFP, American Academy of Otolaryngology-Head and Neck Surgery, AAP; Subcommittee on Otitis Media with effusion, 2004).

1.6.2. Antimicrobial resistance

Antibiotic resistance can be inherent or acquired. Inherent resistance results from the normal genetic, structural, or pathologic state of the microorganisms, while the occurrence of spontaneous mutations or the acquired genetic material encoding a novel resistant mechanism develops in acquired resistance (Low, 2001).

Antibiotic resistance of a microorganism may be caused by several factors, including the production of inactivating enzymes, mutations and post-translational or post-transcriptional modification of the target site, reducing antibiotic affinity to the agent, overproduction of the target of agent, active efflux or reduced uptake of the antimicrobial

agent (Fluit *et al.,* 2001). Repeated exposure of the bacteria to antibiotics and geographical variations may explain the increase in resistance (Pichichero, 2000a).

Beta-lactams are the most extensively used antibiotics (Fluit *et al.,* 2001). Beta-lactams are structurally analogous to peptidyl-D-Ala-D-Ala termini of the peptidoglycan cell wall precursors, responsible for the inhibition of cell wall synthesis. Beta-lactams have the ability to interact with both PBPs and β-lactamase. PBPs are cytoplasmic-anchored enzymes responsible for terminal cell wall synthesis, forming a stable interaction-bond with its target peptidoglycan precursor. Beta-lactamase is chromosomally located or acquired plasmid-encoded enzymes that hydrolyse some β-lactams by forming a labile interaction with the peptidoglycan precursor (Fluit *et al.,* 2001; Low, 2001). Beta-lactamase enzymes are generally produced by *H. influenzae* and *M. catarrhalis,* making them the most important resistant mechanisms to β-lactams and cephalosporins (Sahm *et al.,* 2000; Fluit *et al.,* 2001). The stability of the β-lactam–β-lactamase interaction determines resistance in the target organism. Approximately 200 β-lactamases have been described, with most bacteria containing at least one of the 200 enzymes (Low, 2001).

Streptococcal-resistance to antimicrobials may be a result of an active efflux mechanism, explaining a decreased accumulation of antibiotic within the active site of infection. Bacteria contain an array of cytoplasmic membrane transport systems essential for the uptake of nutrients and the excretion of toxic compounds. These transport systems may play a role in bacteria, by conferring resistance to antibiotics by extrusion of the drug. Some systems handle a wide range of structurally dissimilar compounds, known as multi-drug-efflux-pumps (MDEPs). The majority of MDEPs use a proton motive force as the driving force for efflux. These pumps are divided into three families, v*iz.* the major facilitator superfamily (MFS), the resistance-modulation-cell division family, and small multi-drug-resistant family. Substrates for the MFS pumps in *S. aureus (norA)* and *S. pneumoniae (pmrA)* include fluoroquinolons, and in *S. pneumoniae (mefE)* and *S. pyogenes (mefA)* the 14- and 15-membered macrolides (erythromycin, clarithromycin, and azithromycin) (Low, 2001). Macrolide-resistance in *S. pneumoniae* may present two phenotypes: (1) the M phenotype, encoded by *mef*A, which causes an efflux, thus removing macrolides, and (2) the MLS_B phenotype, encoded by *erm(B)* which produces erythromycin ribosomal methylase and blocks binding of an antimicrobial agent, resulting in resistance (Jacobs, 2004).

Multi-drug-resistance (MDR) is often located on integrons. Integrons are genetic elements consisting of genetic determinants of a site-specific recombination system that captures mobile gene cassettes. The integrons contain integrase and an adjacent

recombination site. The cassettes may be integrated by integrase at the recombination site, and multiple gene cassettes may be present in one intergron. Intergrons are not mobile and are often associated with transposons, well known for their capacity to carry MDR genes. Multi-drug-resistant genes may cause resistance by decreasing the expression of porins and changes in a cell. The changes cause reduced uptake or expression of an efflux (Fluit *et al.,* 2001).

To prevent resistance, an antibiotic is effective when local concentrations greater than the MIC penetrate into the site of infection (Agro, 1999; Jacobs, 2004). Marked geographical differences occur in antibiotic resistance among AOM pathogens and are essential to ensure adequate therapy (Jones *et al.,* 2003).

1.6.3. Complications

Complications are rare in developed countries because of the effective use of administrated antibiotics and low mortality. Antibiotic-resistant pathogens may cause serious and fatal infections (Dagan and Leibovitz, 2002). Since the post-antibiotic era, there has been a major decrease in complications to less than 0.5% (Eneli, 1998). The vast decline favours the use of antibiotics.

Complications occur in three anatomical areas: (1) the aural cavity, including the external, middle, and inner ear, (2) intratemporal, and (3) intracranium (Eneli, 1998). Mastoid dermatitis is a complication of the external ear, while conductive hearing loss, TM perforation, tympanosclerosis, facial nerve palsy, ossicular discomfort, and cholesteatona are middle ear complications. The inner ear is associated with neurosensory hearing loss and suppurative labyrinthitis (Eneli, 1998). Intracranium complications are central nervous system-associated, including petrosis, labyrinthitis, and mastoiditis, while meningitis, extradural- and brain abscess, lateral sinus thrombosis, empyema, and otitic hydrocephalus are intracranium-associated (Eneli, 1998).

Mastoiditis and meningitis are the most common complications, followed by brain abscess (Eneli, 1998; Penido *et al.,* 2005). An otogenic brain abscess is the most life-threatening intracranial complication and is always located adjacent to the temporal bone, leading to osteitis and erosion of the bone (Penido *et al.,* 2005). Meningitis arises from direct invasion via osteothrombophlebitis of small vessels or by labyrinth or endolymphatic channels, or related traumatic bone defects, as a result of simultaneous infection in the URT and middle ear (Eneli, 1998; Aimoni *et al.,* 2005). Intracranial complications seek immediate treatment, which includes carniotomy, drainage of abscess, open mastoidectomy with

abscess drained through the mastoid, open mastoidectomy alone, and closed mastoidectomy (Penido *et al.,* 2005). Meningitis is treated with intravenous antibiotics for 24 to 48 hours after surgery, such as mastoidectomy and spheno-ethmoidectomy (Aimoni *et al.,* 2005). Mastoiditis presents an infra-autricular swelling with chronic otorhea and TM perforation. The disease is difficult to treat with oral antibiotics (Eneli, 1998; Bamberger and Jackson, 2001).

Conductive hearing loss is the most regularly occurring middle ear complication. The degree and frequency of hearing loss (25 to 30 decibels) is associated with an increased mass of fluid in the middle ear, filling the air-filled space and decreasing ossicular motion for sound pressure. A decrease in sound transmission through the middle ear also results in hearing loss (Ravicz *et al.,* 2004). Temporary hearing loss is common in OME, and in the long run may cause bone erosion and retracted pockets (de Ru and Grote, 2004). Conductive hearing loss may lead to behavioural problems and poor academic performances, affecting language and speech. The development of speech and language is important at age three and younger. The effect of OM on concentration, learning and academic achievement is usually not a long-term effect and hearing loss usually restores as OME resolves, unless chronic changes develop (Holm and Kunze, 1963; Eneli, 1998). A Swedish study by Augustsson and Engstand (2001) described no long-term effects of OM upon concentration, learning and academic achievement. However, a Finnish study concluded that many episodes of AOM during the first three years have long-term effects on learning and attention skills, at least up to nine years of age, in spite of active treatment (Loutonen *et al.,* 1998). In New Zealand, SOM was associated with delayed hearing ability up to 15 years of age (Stewart and Silva, 1996).

Surgical complications are commonly found in cases where tympanostomy tube insertion was performed. Two kinds of complications may be present after tube insertion: (1) early complication, occurring while tubes are still in place in the TM, and (2) late complications, occurring after extrusion of the tube. Early complications include persistent otorhea, tube blockage, early extrusion, hearing loss, and ossicular disruption, while late complications include persistent perforation after tube extrusion, scarring of the TM, atrophic monomeric TM, granuloma, tympanosclerosis, cholesteatoma, and migration of the tubes into the ear canal (Pitcher *et al.,* 1997). The use of anaesthesia in surgical procedures is also a risk (Eneli, 1998).

1.7. Other alternatives

1.7.1. Bacterial interference

During the past decade, research has extensively focussed on new alternative treatments in the increasing era of antibiotic resistance. Several strategies have been tested for the treatment of AOM, especially focussed on the protection against OM (Tagg and Dierksen, 2003; Brook, 2005). Bacterial interference (BI) plays an important role in maintaining the normal flora of the upper respiratory tract. Normal flora is maintained by the implantation of indigenous microflora of low virulence, capable of interference, colonization and subsequent infection, with more virulent pathogens. Mechanisms for BI involve colonisation of normal flora on epithelial cells preventing the adhesion of pathogens, changes in the bacterial environment, production of bactericidal substances, and competition for nutritional substances (Brook, 2003; Brook, 2005). Several studies provide evidence that normal microflora of the nasopharynx may support non-specific defence systems against infections such as OM (Fujimori *et al.*, 1996; Brook and Gober, 2000; Tano *et al.*, 2000; Tano *et al.,* 2002). The majority of interfering bacteria include aerobic α-haemolytic streptococci (AHS), mostly *Streptococcus mitis* and *Streptococcus sanguis,* and anaerobic streptococci such as *Peptostreptococcus anaerobius* and *Prevotella melaninogenica* (Bernstein *et al.*, 1993; Bernstein *et al.*, 1994; Brook, 2005).

High numbers of AHS have been reported in the nasopharynx of healthy children compared to those prone to AOM, and are supported by research (Bernstein *et al.,* 1993; Bernstein *et al.*, 1994; Fujimori *at al.,* 1996; Brook and Yocum, 1999; Tano *et al.*, 1999; Brook and Gober, 2000; Walls *et al.*, 2003). AOM prone children tend to have an increase in *S. pneumoniae* and NTHI (Bernstein *et al.*, 1993; Tano *et al.*, 2002). Alpha-haemolytic streptococci isolated from eustachian tube openings have a higher interfering activity against AOM pathogens than those from adenoid tissue (Brook and Yocum, 1999; Tano *et al.,* 1999; Brook, 2005). Tano *et al.* (2002) developed a nasal spray containing AHS with good activity against OM pathogens. However, no difference was obtained in relation to the placebo group. Children with recurrent AOM had less AHS than in healthy children**.** These AHS had a lower inhibitory activity against *S. pneumoniae*, and NTHI to healthy children. This suggests that an isolate of AHS has to be selected with superior adherence to the epithelium of the nasopharynx. None of these children were treated with antibiotics prior to introduction of AHS (Tano *et al.,* 2002). Roos *et al.* (2001) introduced a nasal spray containing five AHS (two *S. sanguinis*, two *S. mitis* and one *S. oralis*) in a double-blind, placebo-controlled study, to reduce recurrent AOM and the frequency of OME. Children were treated with antibiotics for

10 days before the use of either the streptococcal or placebo spray. *In vitro* inhibition against the AOM pathogens was achieved, suggesting the problem involved modification of the normal microflora in the absence of antibiotics (Tagg and Dierksen, 2003).

The decrease in the number of AOM pathogens may be a result of the production of bacteriocins or bacteriocin-like substances (BLIS) that suppress some bacterial growth (Brook and Gober, 1998). *Streptococcus salivarius,* a non-pathogenic bacteria, isolated from the oral cavity of humans, is known to produce salivaricin A and B. Salivaricin A and B and its activity against *S. pyogenes* has been well documented (Dierksen and Tagg, 2000; Walls *et al.*, 2003). *In vitro* inhibitory activity against Gram-negative pathogens such as *M. catarrhalis* and *H. influenzae* was also reported (Walls *et al*., 2003).

De Gutierrez *et al.* (2001) studied the effect of *Lactobacillus fermentum* against *S. pneumoniae* by intranasal introduction. A decrease in the number of *S. pneumoniae* was obtained, together with an increase in the number of activated macrophages and lymphocyte population. An increase in anti-*S. pneumoniae* antibodies also suggested the involvement of the mucosal immune system as a protective barrier. Lactic acid bacteria are known to restore the ecological equilibrium of different mucosal areas (de Gutierrez *et al.*, 2001).

Administration of antibiotics disturbs the balance of natural microflora and facilitates colonization of pathogens (Brook and Foote, 1997; Faden *et al.*, 1997; Brook and Gober, 1998; Brook and Gober, 2005a). Normal oral microflora, such as AHS (mostly *S. mitis* and *S. sanguinis*), anaerobic streptococci (*P. anaerobius*) and *P. melaninogenica*, are generally susceptible to amoxicillin, while these microflora are relatively resistant to second- and third-generation cephalosporin treatment (Brooke and Gober, 2005b). Brook and Foote (1997) studied the effect of amoxicillin and second-generation cephalosporin, cefprozil, on the interfering flora isolated from the adenoids. A decrease in AHS and an increase in the number of *S. aureus* and β-lactamase-producing bacteria were established. No change in AHS was found when treated with cefprozil. Another study compared the effect of amoxicillin/cluvulanate with cefprozil and third-generation cephalosporin, cefnidir. Both amoxicillin/cluvulanate and cephalosporins inhibited the pathogens causing AOM. The oropharyngeal bacteria were more depleted by amoxicillin/cluvulanate than after treatment with cephalosporins (Brook and Gober, 1998; Brook and Gober, 2005b).

Various strains of the normal nasopharyngeal flora produce bacteriocins or BLIS, and may be used as an alternative to antibiotics. A few advantages and disadvantages of an interfering organism are listed in Table 2.

Table 2: Advantages and disadvantages of a BLIS-producing strain when used in replacement therapy (modified from Tagg and Dierksen, 2003).

Advantages
1. Narrow spectrum of activity
2. Cost effective, long-term protection against specific infections
3. Direct implantation, thus highly competitive
3. Foster increased protection by natural transmission through close contact with host
4. Genetic engineering to obtain a superior strain
Disadvantages
1. As a result of quantitative and qualitative stability of normal flora, invasion of any foreign microbe may be limited
2. High intrinsic stability of indigenous flora may be a major obstacle in modification of the composition by introducing a specific effector strain
3. Effector strain has to be strongly competitive; thus it must be administrated before microbial climax community is established or after exposure to antimicrobial agents to create an appropriate niche. Once antibiotic selective pressure is removed, resistant strains may disappear

Several principle characteristics are required for interfering strains to be used in replacement therapy. These include: specific activity against pathogens, avoiding disruption of normal microflora, the ability to survive a selected habitat and not elsewhere, non-pathogenic or weak opportunistic pathogens for host, susceptibility to low-risk antibiotics, easy to prepare for commercial distribution, easy to identify among resistant microflora, not causing cytotoxicity of immunological sensitisation in host, and persisting in host tissue to effect long-term protection (Tagg and Dierksen, 2003).

1.7.2. Vaccines

Little research is done on the development of specific vaccines against OM. For an OM vaccine to be accepted, it has to be safe and efficient, induce an appropriate immune response to clear pathogens and decrease colonisation in the nasopharynx, without causing any immune-mediated damage to the middle ear (Cripps *et al.,* 2005). Otitis media is a polymicrobial disease, which complicates the development of vaccines. To prevent OM, vaccines have to inhibit *S. pneumoniae,* NTHI, *M. catarrhalis,* RSV and parainfluenza. A number of lead protein antigens for all three bacterial pathogens have been identified (Cripps *et al.,* 2005; Murthy *et al.,* 2005).

Pneumococcal vaccines do not have a significant impact on the prevalence of OM (Cripps *et al.,* 2005). Pneumococcal vaccines include pneumococcal polysaccharide and pneumococcal conjugate vaccines, both effective against pneumococcal disease. *S. pneumoniae* are an encapsulated bacteria consisting of complex polysaccharides, pathogenic to humans. Based on the antigenic properties of the capsules, the bacteria may be classified into various serological groups. About 90 serotypes have been identified (Reinert, 2004; Sokos, 2005). The current pneumococcal polysaccharide vaccine consists of 23 capsular polysaccharide antigens representing 85-90% of serotypes responsible for invasive pneumococcal infection. Six of the 23 antigens cause drug-resistant diseases. Pneumococcal polysaccharide vaccines cause T-cell independent immune responses and can only activate mature B-cells (Reinert, 2004; Sokos, 2005). Pneumococcal conjugant vaccines are T-cell dependent antigens providing protection against only a limited number of serotypes (Reinert, 2004).

The heptavalent pneumococcal conjugate vaccine (PvCV7) was designed based on serotypes predominant in the USA. The heptavalent pneumococcal conjugate vaccine was the first pneumococcal vaccine approved for use in children younger than 2 years of age (van Kempen *et al.* 2005). Although PvCV7 is effective in the eradication of pneumococcal disease, only a slight decrease in OM was observed. A study conducted in Finland evaluated the efficacy of the vaccine in children with AOM. A 34% and 57% reduction in respiratory and pneumococcal infections was demonstrated, respectively, with only a 6-7% reduction of AOM. A 33% increase in episodes caused by serotypes, not included in the vaccine, with an 11% increase in OM infection caused by *H. influenzae*, was also observed (Eskola and Kilpi, 1999; Eskola *et al.,* 2001; Fireman *et al.,* 2003). A shift in *S. pneumoniae* serogroups from the conjugate vaccine to non-vaccine serotypes was noted following vaccination (van

Kempen *et al.,* 2005). In cases where high-dose amoxicillin therapy and pneumococcal conjugate vaccinations were used, *H. influenzae* has become the predominant pathogen. Fewer occurrences of penicillin-resistance *S. pneumoniae* AOM isolates and more beta-lactamase-producing strains of *H. influenzae* were recorded (Casey and Pichichero, 2004). Other studies indicated that the immunisation of infants with PcCV7 resulted in a 20.1-24% less occurrence of ventilation tubes for recurrent AOM than unimmunised controls (Eskola *et al.,* 2001; Fireman *et al.,* 2003). In an Israeli study carried out with toddlers attending day care, a 20% decrease in AOM was observed when associated with antibiotics, with a decrease in antibiotic resistant *S. pneumoniae (*Dagan *et al.,* 2001; Dagan *et al.,* 2003). Serotype coverage greatly differs between countries, age and disease. The serotype also varies in virulence, depending on the ability to activate the alternative pathway to alter resistance to phagocytosis (Reinert, 2004).

Little research has been done on the development of vaccines against NTHI and *M. catarrhalis* in OM (Murthy *et al.,* 2005). Although a conjugate *H. influenzae* type b vaccine has been used, no effect on the prevalence of OM was recorded (Cripps *et al.,* 2005). Vaccines for viruses do exist but do not seem to affect OM. The influenza vaccine had a slight effect on the prevention of OM, but it is not practical to include in a polymicrobial otitis vaccine because of its active constituents (Murthy *et al.,* 2005). The impact of existing respiratory syncytial virus vaccines on OM has not been studied. Vaccines developed for parainfluenzae had no effect on the eradication of AOM (Cripps *et al.,* 2005).

The Centers Committee and Immunization Practices recommend universal influenza vaccine immunization for all children six months and two years of age (AAP and AAFP, Clinical Practice Guidelines; Subcommittee on Management of Acute Otitis Media, 2004). However, due to a lack in scientific data, the application of the influenza vaccine in AOM is limited (Reinert, 2004).

2. The genus *Enterococcus*

2.1. Introduction

The genus *Enterococcus* belongs to a group of generally regarded as safe (GRAS) microorganisms, known as lactic acid bacteria (LAB) (Devriese and Pot, 1995). Enterococci are Gram-postitive, catalase- and oxidase-negative, non-sporeforming, facultative anaerobic

cocci (Holt, 1994; Moreno *et al.* 2005). Enterococci are ubiquitous LAB, isolated from various sources including fermented meat, olives, vegetables and diary products. They form part of the natural microflora of the gastrointestinal tract (GIT) of animals and humans (Murray, 1990; Franz *et al.,* 1999; Moreno *et al.,* 2005). Some strains cause food spoilage and have been associated with diseases (Franz *et al.,* 1999). Over the past two decades, enterococci have emerged as potential opportunistic pathogens in nosocomial disease, becoming a serious problem in human medicine as a result of their increase in antibiotic resistance, and the production of various virulence determinants (Franz *et al.,* 1999; Franz and Holzapfel, 2004).

2.2. Biochemical, physiological and phenotypic characterization

Lactic acid bacteria are grouped into different genera based on various morphological, physiological and biochemical characteristics. Lactic acid bacteria can be divided into rods (*Carnobacterium* and *Lactobacillus*) and cocci (all other genera). One exception is the genus *Weissella,* which includes cocci and rods. The tetrad-forming genera are *Aerococcus, Pediococcus* and *Tetragenococcus* (Axelson, 2004).

Enterococcus was first described as a diplococcus by Thiercelin and Jouhaud in 1899 (Thiercelin and Jouhaud, 1903). In 1906 it was reclassified as *Streptococcus faecalis* based on the formation of short and long chains (Andrews and Horder, 1906). However, the genus *Enterococcus* was separated from *Streptococcus* based on 16S rRNA cataloguing, DNA:DNA and DNA:rRNA hydridisation, and serological studies with superoxide dismutase antisera (Schleifer and Kilpper-Bälz, 1984). Enterococci form a distinct cluster with *Vagococcus, Tetragenococcus* and *Carnobacterium* in the clostridial sub-branch (Devriese *et al.,* 1993; Franz *et al.,* 1999). The phenotypic description of the genus *Enterococcus* is vague, if not impossible (Devriese *et al.,* 1993; Devriese and Pot, 1995). Identification relies on the use of a combination of phenotypic, genotypic, and phylogenetic information in a polyphasic taxonomic approach, as described by Vandamme *et al.* (1996). Currently, 28 species are assigned to the genus, which is subdivided into at least seven recognised species based on 16 S rRNA gene sequences (Stiles and Holzapfel, 1997; Franz and Holzapfel, 2004). Species are differentiated mainly by carbohydrate fermentation patterns, arginine and hippurate hydrolysis, and the presence and/or type of menaquinones (Schleifer and Kilpper-Bälz, 1987). Most enterococci are positive to the Voges-Proskauer test and fermentation of ribose (Franz and Holzapfel, 2004). Enterococci are chemo-organotrophs with a strictly fermentative metabolism, using glycolysis to convert glucose quantitatively to

lactic acid (Franz and Holzapfel, 2004). The phylogenetic relationship has been determined by comparative sequence analysis of the 16S rRNA and is divided into seven groups (see Table 3).

Table 3: Characteristic physiological properties and phylogenetic groups of enterococci.[a, b]

	Growth at		Growth in the presence of					
Phylogenetic groups and species	10 ºC	45 ºC	pH 9.6	6.5% NaCl	40% Bile	0.04% Sodium azide	Esculin hydrolysis	Group D antigen
***E. faecium* group**								
E. azikeevi	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
E. canis	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
E. faecium	+	+	+	+	+	+	+	V
E. durans	+	+	+	+	+	+	+	(+)
E. hirae	+	+	+	+	+	+	+	V
E. mundtii	+	+	+	+	+	+	+	+
E. villorum	n.d	n.d.	n.d.	+	+	+	+	n.d.
***E. avium* group**								
E. avium	V	+	+	V	v/+	n.d.	+	+
E. malodoratus	+	-	+	+	+	n.d.	+	+
E. pseudoavium	+	+	+	+/-	V/+	n.d.	+	-
E. raffinosus	(+)	+	+	+	V/+	n.d.	+	n.d.
E. gilvus	+	+	n.d.	+	+	n.d.	+	+
***E. gallinarium* group**								
E. gallinarium	+	+	+	+	+	+	+	+
E. casseliflavus	+	+	+	V/+	+	+	+	+
E. flavescens	V/-	V/+	n.d.	+	+	+	+	+
***E. dispar* group**								
E. dispar	+	-	n.d.	+/-	+	-	+	-
E. asini	(+)	(+)	n.d.	-	+	n.d	+	+
E. pallens	+	+	n.d.	+	+	n.d.	+	+
***E. saccharolyticus* group**								
E. saccharolyticus	+	+	n.d.	(+)	+	n.d.	+	-
E. sulfureus	+	-	n.d.	+	+	n.d.	+	-
***E. cecorum* group**								
E. cecorum	-	+	(+)	-	(+)	-	+	-
E. comlumbae	-	n.d.	n.d.	-	(+)	-	+	-
***E. faecalis* group**								
E. faecalis	+	+	+	+	+	+	+	+
E. haemoperoxidus	+	-	n.d.	+	+	+	+	+
E. moraviensis	+	-	n.d.	+	+	+	+	+
E. ratti	+	+	n.d.	+	n.d.	n.d.	+	(+)
Other								
E. phoeniculicola	n.d	n.d	n.d	-	-	n.d.	n.d.	n.d.

Table 3: (continued)

Phylogenetic groups and species	Growth at		Growth in the presence of					
	10 ºC	45 ºC	pH 9.6	6.5% NaCl	40% Bile	0.04% Sodium azide	Esculin hydrolysis	Group D antigen
E.porcinus	+	+	n.d.	+	n.d.	n.d.	+	+
E.solitarius	+	+	n.d.	+	+	n.d.	+	+

[a] Franz and Holzapfel, 2004, [b] Moreno *et al.,* 2005. n.d. = not determined, (+) = weak positive, V = variable, +/- = differing reports in literature.

Enterococcus spp. grow between 10 ºC and 45 ºC, in the presence of 6.5% NaCl, and between pH 4.4 and 9.6. All species belong to Lancefield antigen group D (Prescott *et al.,* 1999). *Enterococcus sulfureus, Enterococcus palens, Enterococcus gilvus, Enterococcus mundtii* and *Enterococcus casseliflavus* produce a yellow-pigment. *E. casseliflavus* and *Entoerococcus gallinarum* are motile (Moreno *et al.,* 2005). Genotypic methods used to differentiate enterococci and lactococci on genus level involves PCR with conserved 16S rRNA gene sequences (Deasy *et al.,* 2000). Enterococci are differentiated by PCR amplification of a conserved internal fragment of D-alanine:D-alanine ligase genes, by restriction fragment length polymorphism of the 16S/23S intergenic spacer region, tRNA intergenic spacer PCR and RAPD-PCR (Tyrrell *et al.,* 1997; Baele *et al.,* 2000; Ozawa *et al.,* 2000; Franz and Holzapfel, 2004).

2.3. Infections caused by enterococci

Enterococci are the most common nosocomial pathogens in immunocompromised patients with structural abnormalities (Chenoweth and Schaberg, 1990; Murray, 1990; Moellering, 1992; Morrison *et al.*, 1997). Enterococci are responsible for 12% of infections, and are the second greatest cause of hospital-acquired infections in the USA (Schaberg *et al.*, 1991). These bacteria are typical opportunistic pathogens, and have been an important cause of bacteremia, infective endocarditis, urinary tract infections (UTI), infections of the central nervous system, intra-abdominal and pelvic infections and wound infections (Franz *et al.,* 1999). *Enterococcus faecalis* and *Enterococcus faecium* are responsible for up to 90% and 10% of infections, respectively. *Enterococcus avium, E. casseliflavus, Enterococcus durans, E. gallinarum, Enterococcus hirae, Enterococcus malodoratus, E. mundtii, Enterococcus pseudoavium, Enterococcus raffinosus and Enterococcus solitarius* are only occasionally associated with infections (Mundy *et al.*, 2000). Enterococci consist of virulent

factors and are resistant to antimicrobial agents that make them effective opportunistic pathogens (Moreno *et al.,* 2005).

2.4. Antibiotic resistance

Enterococci from clinical, food and faecal sources are resistant to a broad range of antibiotics (Leclercq *et al*., 1992; Moellering, 1992). Enterococci are either intrinsically resistant or they possess acquired resistance determinants. Intrinsic resistant genes are located on the chromosome, while acquired resistant genes are located on plasmids or transposons (Clewell, 1990; Murray, 1990). Constitutive intrinsic resistance was described for cephalosporins, β-lactams, sulphonomides, low levels of clindamycins and aminoglycosides. Acquired resistance includes resistance to ampicillin, macrolides, trimethoprim/sulfomethoxole, quinolones, streptogramins, chloramphenicol, erythromycin, high levels of clindamycins and aminoglycosides, tetracycline, high levels of β-lactams, fluoroquinolons and glycopeptides, such as vancomycin (Murray, 1990; Leclercq, 1997; Morrison *et al.,* 1997; Moreno *et al.,* 2005).

Infections with multiple-antibiotic resistant *Enterococcus* strains are normally treated with clinically relevant antibiotics such as ampicillin, gentamycin and vancomycin (Franz and Holzapfel, 2004). As result of the extensive use of vancomycin, high levels of vancomycin-resistant enterococci (VRE) have emerged (Endz *et al.,* 1999). VRE possess *van*A, *van*B, *van*D, and *van*E type resistance genes, with *van*A and *van*B considered to be of clinical importance (Moreno *et al.*, 2005).

Many cases of resistance are located in mobile genetic elements, such as conjugative transposons and resistant plasmids typical for enterococcal species (Noble *et al.,* 1992). The spread of antibiotic resistance, via these elements to other genera, such as pathogenic *Staphylococcus*, may be of major concern. Vancomycin-resistant transposons of enterococcal strains, involved in nosocomial infections, have received a great deal of attention (Noble *et al.,* 1992). Tn916 was the first enterococcal transposon described conferring resistance to tetracycline. Tn916 is expressed in both Gram-positive and Gram-negative bacteria, and is used in genetic studies to inactivate genes in recipient strains (Franke and Clewell, 1981; Gasson, 1990). Conjugation is the transfer of DNA from a donor cell to the recipient by direct cell-to-cell contact. In LAB, donor-recipient pairs are formed by the production of sex pheromones, or substances produced by the recipient that stimulates cell aggregation (Clewell *et al.,* 2002). Examples of conjugative plasmids with a broad host

range are pAMβ1 (Clewell *et al.,* 1974) and pIP501 (Horodniceanu *et al.,* 1976), encoding erythromycin-resistance and chloramphenicol-erythromycin resistance, respectively. These plasmids are regularly conjugative to other genera of LAB (Gibson *et al.,* 1979; Gonzalez and Kunka, 1983).

The classical regimen for treating systemic enterococcal infections is a synergic combination of a cell-wall-active antibiotic (penicillin or ampicillin) and aminoglycoside, which is ineffective in the presence of resistance to one or both components (Witte, 1999; Moreno *et al.,* 2005). To use enterococci as potential starter cultures, it is necessary to check for the absence of transferable antibiotic resistance, as it is strain specific (Moreno *et al.,* 2005).

2.5. Virulence factors

Virulence of enterococci cannot be explained by antibiotic resistance alone. To cause infection, enterococci have to consist of various virulent determinants enhancing colonisation, invasion and translocation of the strain to host tissue and epithelial cells (Johnson, 1994; Moreno *et al.,* 2005). Virulent traits of enterococci include adherence to host tissue by a variety of adhesions, e.g. the enterococcal surface protein (Esp), aggregation substance (AS), enterococcal endocarditis antigens ($EfaA_{fm}$ or $EfaA_{fs}$ from *E. faecium* and *E. faecalis*, respectively), or adhesion to collagen (Ace). Enterococcal virulence is also based on invasion and abscess formation, resistance to and modulation of specific and unspecific host defence mechanisms, and by the production and secretion of toxins such as the plasmid-encoded cytolysin, and secretion of hyaluronidase and gelatinase, which may result in pathological changes (Witte*,* 1999; Franz and Holzapfel, 2004; Moreno *et al.,* 2005).

2.5.1. Aggregation substance (AS)

An aggregation substance is a pheromone-inducible surface-bound glycoprotein produced by *E. faecalis* and is encoded on a sex pheromone-responsive plasmid. The AS mediates mating aggregate formation during conjugation, which is the binding of donor cells to plasmid-free recipients, thereby facilitating plasmid transfer. The AS is essential for high-efficiency conjugation of sex pheromone plasmids (Mundy *et al.,* 2000; Semedo *et al.,* 2003; Moreno *et al.,* 2005). The AS has several other functions, including adherence to a variety of eukaryotic tissue surfaces, such as pig tubular cells, influences phagocytosis and the subsequent fate of an organism, and contribute to pathogenesis of enterococcal infections by a number of mechanisms (Mundy *et al.,* 2000; Sübmuth *et al.,* 2000; Semedo *et al.,* 2003; Moreno *et al.*, 2005). It is speculated that macrophages act as a vehicle of enterococci from

the intestine to the lymph system and bloodstream, thus facilitating transport (Wells *et al.,* 1990). The AS may work synergistically with cytolysin in endocarditis (Mundy *et al.,* 2000). The AS is also associated with adhesion (adhesins) to eukaryotic cells, promoting colonization, invasion of eukaryotic cells, and adhesion to extracellular matrix protein to promote translocation, and increase the survival in immune cells by evading the host immune system (Franz and Holzapfel, 2004).

2.5.2. Sex pheromones

Sex pheromones are small, linear, peptides of 7-8 amino acids, excreted to promote acquisition of plasmid DNA and cause pathological changes such as acute inflammation. When pheromones bind to receptors on the cell surface of strains containing plasmid DNA, a signal is transducted and leads to induction of the AS gene. Expressed peptide mediates cell clump formation by binding to a complementary receptor, allowing a highly efficient transfer of the pheromone plasmid on which the AS gene is encoded. Sex pheromones are associated with surface proteins of unknown function. Sex pheromones may also serve as chemo-attractive substances for human neutrophils and induce inflammation and superoxide production (Franz and Holzapfel, 2004).

2.5.3. Cytolysin (Cyl)

Cytolysin is an eukaryotic toxin and lyses the immune cells to invade the immune response (Franz and Holzapfel, 2004). Cytolysin was the first-studied virulence factor, and may be encoded either on a highly transmissible pheromone-responsive conjugative plasmid, or integrated into bacterial chromosome or pathogenicity islands (Mundy *et al.,* 2000; Semedo *et al.,* 2003; Moreno *et al.,* 2005). Cytolysin is a unique novel post-translationally-modified bacterial protein that is distantly related to lantibiotic bacteriocins, contributing to the severity of disease, such as bacteremia and systemic disease (Booth *et al.,* 1996). The toxin causes a β-hemolytic reaction on red blood cells and has an activity against a large variety of Gram-positive bacteria (Mundy *et al.,* 2000; Semedo *et al.,* 2003). Cytolysin is also responsible for direct tissue damage by a reduction in renal function and complete destruction of the renal architecture (Chow *et al.,* 1993; Booth *et al.,* 1996). In some cases the toxin is responsible for organotoxic activity, where combined effects of antimicrobial and anti-inflammatory therapy are set off and the organ is completely destroyed (Mundy *et al.,* 2000). Cytolysin is generally isolated from *E. faecalis, E. durans* and *E. faecium*, with 60% of *E. faecalis* strains isolated from disease outbreaks (Mundy *et al.,* 2000; Semedo *et al.,* 2003). *E. faecalis* isolated from endocarditis and bacteremia has the ability to adhere to Girarti heart cell lines (Archimbaud *et al.,* 2002; Moreno *et al.,* 2005).

2.5.4. Enterococcus surface protein from E. faecalis *(*Esp_{fs}*) and* E. faecium *(*Esp_{fm}*)*

Enterococcus surface proteins are large, chromosomally gene-encoded adhesions, consisting of an interesting structure mostly produced by *E. faecalis* (Moreno *et al.,* 2005). The central part consists of distinct repeated regions acting as a retractable arm with an extended globular N-terminal through the cell to the surface. During adverse conditions, such as immune deficiencies, the ability to retract surface proteins may facilitate immune evasion (Mundy *et al.,* 2000). Esp is associated with the ability to form biofilms at abiotic surfaces such as stainless steel and infections involving catheters (Semedo *et al.,* 2003). Biofilms generally induce antimicrobial and antibiotic resistance. *Enterococcus* surface proteins contribute to colonization and persistence of enterococci in urinary tract infections, associated with colonisation and spread among liver transplant patients, and is part of the pathogenicity island in *E. faecalis* (Shankar *et al.,* 2001; Toledo-Arana *et al.,* 2001; Eaton and Gasson, 2002; Shankar *et al.,* 2002; Waar *et al.,* 2002; Semedo *et al.*, 2003). *Enterococcus* surface proteins exhibit microbial surface components recognizing adhesive matrix molecules (MSCRAMM) characteristics with a role in adhesion and evasion of immune response (Franz and Holzapfel, 2004; Moreno *et al.,* 2005).

2.5.5. Adhesin to collagen from E. faecalis *(*Ace*) and* E. faecium *(*Acm*)*

The adhesins *Ace* and *Acm* are responsible for pathogenesis in enterococci. Adhesion to collagen is generally expressed during human infection, promoting translocation and evasion of the host immune system (Franz and Holzapfel, 2004).

2.5.6. Enterococcus endocarditis antigen from E. faecalis *(*$EfaA_{fs}$*) and* E. faecium *(*$EfaA_{fm}$*)*

The production of these antigens is important in the infection of human tissue, endocarditis (Franz and Holzapfel, 2004). It suggested that a carbohydrate antigen mediates the adherence of enterococci to cultured heart cells (Guzman *et al.,* 1991a).

2.5.7. Gelatinase (Gel)

Gelatinase is an extracellular Zn-metalloprotease capable of hydrolysing various proteins such as collagen, casein, gelatine and other small biologically-active peptides, to evade the host innate response, resulting in an increase in pathogenicity (Mundy *et al.,* 2000; Franz and Holzapfel, 2004; Moreno *et al.,* 2005). The majority of Gel is produced by *E. faecium* and *E. faecalis*, but it is also found in *E. avium, E. casseliflavus, E. durans* and *E. raffinosus* (Semedo *et al.,* 2003; Moreno *et al.,* 2005). Cleavage of hydrolysed peptides by Gel signifies

the potential to modulate the host response to enterococcal infection (Dupont *et al.,* 1998). Gelatinase is also important in translocation. The genes encoding for Gel are normally located on the chromosome (Semedo *et al.,* 2003).

2.5.8. Hyaluronidase

Hyaluronidase is a cell-surface-associated enzyme responsible for cleavage of the mucopolysaccharide moiety of connective tissue or cartilage, and degrades hyaluronix acid, a major component in the extracellular matrix, for the translocation and pathogenesis of some *Enterococcus* species (Jett *et al.,* 1994; Witte, 1999; Franz and Holzapfel, 2004). Strains producing hyaluronidase are generally isolated from abscesses and play a role in host tissue invasion (Jett *et al.,* 1994). The enzyme also acts as a spreading factor for dissemination of some microorganisms (Witte, 1999).

2.5.9. Lipoteichoic acid (LTA)

Lipoteichoic acid, also known as a group D streptococcal antigen, is present in a variety of biological processes. Some properties ascribed to it include modulation of the host immune response and mediating the adherence of enterococci to host cells (Guzman *et al.,* 1989; Guzman *et al.,* 1991a; Guzman *et al.,* 1991b; Montravers *et al.,* 1997). Lipoteichoic acid induces the production of interleukin-1β, interleukin-6 and TNF-α *in vitro* and may contribute to local tissue damage (Witte, 1999).

2.5.10. Extracellular superoxide

Strains isolated from bacteremia are known to produce superoxide. The vast majority of *E. faecalis* produce superoxide, but it varies among strains. The role of superoxide in pathogenesis is not well defined. Strains capable of producing superoxide may be better adapted physiologically to utilise limited sources in the intestinal environment, leading to overgrowth of the organism. Production of superoxide may enhance niche control in proximity to the intestinal epithelium. The effect of membrane damage by oxygen radicals may then potentiate the ability of the organism to translocate across the weakened epithelial barrier (Huycke *et al.,* 1996). Superoxide enhances the *in vivo* survival of *E. faecalis* in mixed infections with *Bacteriodes fragilis* in a subcutaneous infection model (Mundy *et al.,* 2000).

3. Bacteriocins of genus *Enterococcus*

3.1. Introduction

Bacteriocins are small, cationic, amphiphilic (rather hydrophobic) biologically active proteinaceous compounds, exhibiting antimicrobial properties, usually against closely related bacterial species, including food-borne and spoilage organisms (Klaenhammer, 1993; De Vuyste and Vandamme, 1994; Ennahar *et al.,* 2000). *Enterococcus* species produce bacteriocins, known as enterocins. Enterocins are microbial active peptides and have developed a great deal of interest as an approach to control food-borne diseases, to be used as starter cultures and biopreservative in various food products. In some cases enterococci are used as probiotics as a result of their protective effects in the GIT (De Vuyst and Vandamme, 1994; Franz *et al.,* 1999).

3.2. Classification

Bacteriocins are classified into four major classes based on their structural, physiochemical and molecular characteristics, and bactericidal activity (Klaenhammer, 1993; Nes *et al.,* 1996; Moll *et al.,* 1999). Enterococci are known to produce class II bacteriocins. These antimicrobial peptides are small (<10 kDa), heat-stable, cationic, hydrophobic and membrane-active peptides (De Vuyst and Vandamme, 1994; Abee *et al.,* 1995; Nes *et al.,* 1996; Rodriguez *et al.,* 2003). Class II, the largest group, is further classified into three subgroups based on their primary structures. Class IIa or pediocin-like bacteriocins are characterised by the presence of an unique highly conserved hydrophilic N-terminal amino acid sequence, YGNGVXaaC (Tyr-Gly-Asn-Gly-Val- Xaa-Cys) consensus motif, containing a high content of nonpolar amino acid residues, small amino acids (e.g. glycine), with one or more disulphide bridges, and strong inhibitory activity towards *Listeria.* The hydrophobic and/or amphiphilic C-terminal is also moderately conserved with low similarity (Nes *et al.,* 1996; Eijsink *et al.,* 1998; Ennahar *et al.,* 2000). Class IIb bacteriocins consist of a two-component system, where activity depends on two distinct peptides with no significant similarities. Class IIc bacteriocins are thiol-activated and *sec*-dependent secreted miscellaneous peptides requiring a reduced cysteine residue for activity (Abee *et al.,* 1995; Ennahar *et al.,* 2000; Héchard and Sahl, 2002). Class I bacteriocins or lantibiotics are small (<5 kDa), cationic, heat-resistant peptides, consisting of unusual and special amino acids, lanthionine and β-methyl-lantionine, with thioether rings and dehydrated residues. These membrane-active peptides are synthesised via post-translational modifications to inhibit

sensitive organisms (Klaenhammer, 1993; Moll *et al.,* 1999). Class III bacteriocins are large, hydrophilic (>30 kDa), heat-labile peptides and may furthermore include enzymes mimicking their physiological activities (Klaenhammer, 1993; Moll *et al.,* 1999). Class IV bacteriocins are complex peptides, where activity depends on the composition of the protein plus a lipid or carbohydrate moiety. Class IV bacteriocins are not generally accepted (Klaenhammer, 1993). To date, no enterocin has been classified into classes IIb, III, and IV. Class II is the most abundant and extensively studied of bacteriocins (Franz and Holzapfel, 2004).

3.3. Biosynthesis and genetic organisation

Four genes are required for the production of antimicrobial activity of class II bacteriocins. These genes are similarly organised in an operon cluster and may be encoded on the chromosome, or a pheromone-responsive, conjugate plasmid, or a transposon (Gilmore *et al.,* 1994; Martinez-Bueno *et al.,* 1994; Tomita *et al.,* 1996; Chen and Hoover, 2003). The four genes consist of: (1) a structural gene encoding for a prepeptide containing a conserved leader peptide cleaved at a double-glycine proteolytic processing site, (2) a cationic immunity gene protecting the producer against its own bacteriocin, and (3 and 4) genes encoding a membrane-associated ATP-dependent binding cassette (ABC) transporter and an accessory protein essential for externalisation of class II leader peptides. The majority of immunity genes are cytoplasm-associated, with a small part interacting with the cytoplasmic membrane. In some cases regulatory genes are produced (Håvarstein *et al*., 1995; Nes *et al.,* 1996; Chen and Hoover, 2003). Figure 2 illustrates the genetic organization of enterocin CRL353 (Saavedra *et al*., 2004), enterocin SE-K4 (Doi *et al.,* 2002), enterocin P (Cintas *et al.,* 1997) and enterocin A (O'Keeffe *et al.,* 1999). Enterocin CRL35 and mundticin KS share similar genetic clusters.

Bacteriocins of LAB are ribosomally synthesised as N-terminal precursor proteins containing a leader sequence cleaved during maturation (Nissen-Meyer and Nes, 1997). The leader peptides consist of highly conserved sequences recognised by a proteolytic domain of a dedicated ABC-transporter superfamily, and are secreted via a signal-sequence-independent pathway (Klaenhammer, 1993; Nes *et al*., 1996). Class IIa bacteriocins are ribosomally synthesised as a prepeptide with a N-terminal leader peptide of a double-glycine type processing site that is secreted out of the cell by transport machinery, except enterocin P (Cintas *et al.,* 1997). Enterocin P is secreted via the general secretory pathway of a cell, formally described as class IIc (Ennahar *et al.,* 2000; Cleveland *et al.,* 2001). Recently, a *sec*-dependent class IIa bacteriocin has been discovered (Cintas *et al.,* 1997). In class IIa

bacteriocins the N-terminal and C-terminal parts of the ABC-transporters are a priority. The C-terminal has a highly conserved ATP-binding domain, while the hydrophobic integral membrane N-terminal domain is responsible for the proteolytic cleavage of the C-terminal part of the double-glycine motif removing the leader. Binding of the prepeptide triggers ATP hydrolysis, resulting in subsequent conformational changes, which is then followed by translocation across the membrane (Nes *et al.,* 1996; Ennahar *et al.,* 2000). ABC-transporters require ATP to transport the bacteriocin (Moll *et al.,* 1999).

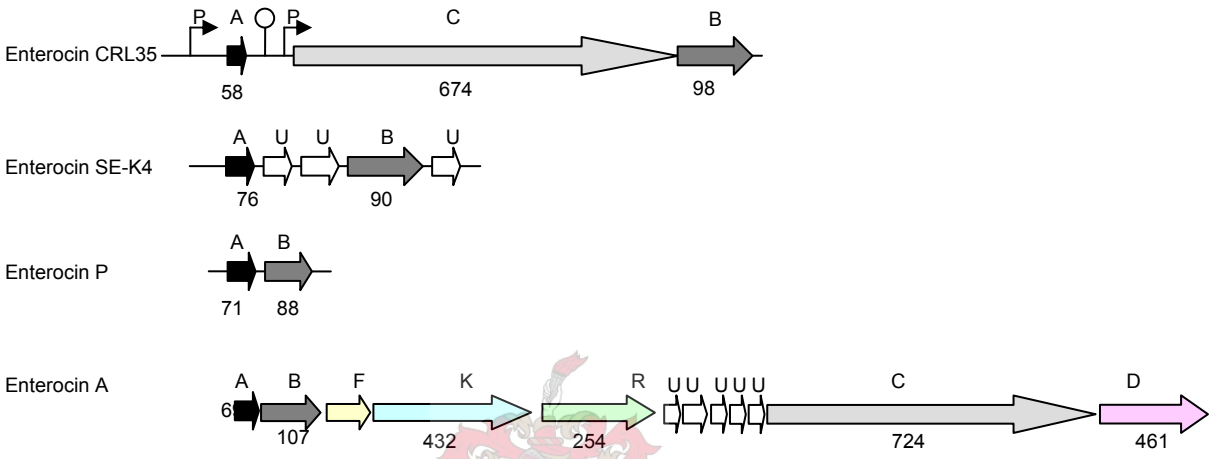


FIG. 2. Genetic organization of enterocin CRL35, enterocin SE-K4, enterocin P and enterocin A. Open reading frames (ORFs) encoding the following proteins are marked by arrows with the corresponding shading patterns: Prepeptide (A); immunity protein (B); ABC-transporter (C); accessory protein (D); induction factor's prepeptide (F); histidine protein kinase (K); response regulator (R); product with unidentified function (U) ; putative promoter (P) ; and transcriptional terminator (T). The number of amino acids residues within each protein is shown below the corresponding ORF.

Biosynthesis of class IIa bacteriocins is regulated by a 3-component system, including: (1) a membrane-bound histidine protein kinase (HPK), (2) an induction factor (IF) and (3) a cytoplasmic response regulator (RR). For biosynthesis, a prebacteriocin and a bacteriocin-like prepeptide of an induction factor are produced. The IF is a small, hydrophobic synthesised peptide acting as an external signal for gene transcription of bacteriocin genes. The bacteriocin and IF are released via the ABC-transporters and are sensed by the HPK. The histidine residue in the extracellular domain autophosphorylates and is transferred to a conserved aspartic acid of the RR receiver. The interaction causes intramolecular changes, triggering the RR to activate transcription of regulatory genes, including the structural, externalising and immunity genes (Chen and Hoover, 2003).

3.4. Mode of bacteriocin activity

Activity depends on the structure of a bacteriocin, and the composition and amount of cytoplasmic membrane (Héchard and Sahl, 2002). Bacteriocins produced by LAB interact with specific targets in the cytoplasmic membrane making it more permeable through the formation of complex pores, resulting in the leakage of inorganic phosphate and an ionic imbalance (Ennahar *et al.*, 2000). The consequence of such disturbances is the dissipation of the proton motif force (PMF), including partial or total dissipation of either or both the transmembrane potential and/or the pH gradient.

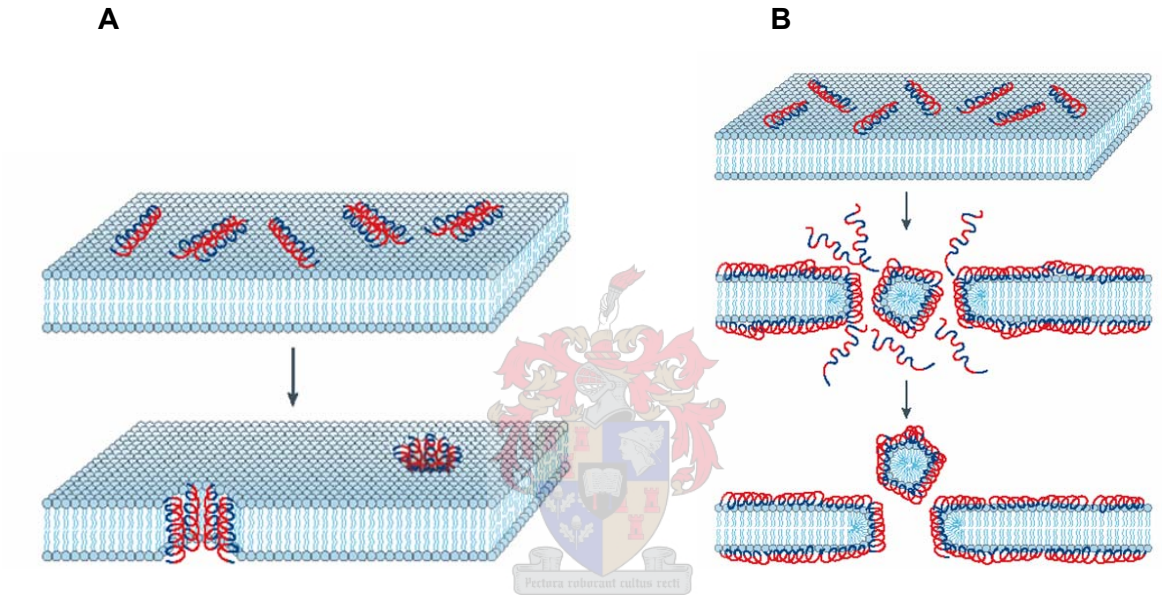


FIG. 3. Class II bacteriocin insertion and pore formation. A). The barrel-stave model. Peptides attach to the membrane via electrostatic- and hydrophobic interactions, causing aggregation and insertion into the bilayer so that the hydrophobic peptide regions align with the lipid core region and the hydrophilic regions form the interior region of the pore; B). The carpet-like model. Membrane disruption is caused when the peptide is orientated parallel to the surface of the lipid bilayer, forming an extensive layer or carpet. Hydrophilic and hydrophobic regions of the peptide are shown in red and blue, respectively (Brogden, 2005).

Class II bacteriocins have a broad spectrum of activity with post-translational modifications before exported from cell. The activated bacteriocins interact with various membrane-bound target molecules to form large and non-specific pores, causing total dissipation of both the transmembrane potential and the pH gradient. Dissipation of the PMF mediates the efflux of ions, amino acids and ATP from cells and liposomes (Moll *et al.,* 1999; Héchard and Sahl, 2002). Class IIa bacteriocins provoke a total dissipation of the pH gradient and only partially the transmembrane potential (Ennahar *et al.*, 2000). Proton efflux

is responsible for strong cytotoxic effects, resulting in a decrease in intracellular pH, inhibiting many enzymatic processes (Moll *et al.,* 1999). Pore formation in class IIa bacteriocins may be explained by mimicking of the 'barrel stave' model or the 'carpet-like' model (Fig. 3) (Brogden, 2005). The barrel-stave model pores are formed as a result of the peptides' amphiphilic transmembrane helices, water solubility and membrane-binding ability (Fig. 3A). The initial step in pore formation is activated in two ways, namely: (1) electrostatic interaction between the positively charged amino acids and heads of the phospholipids in the bilayer, and (2) hydrophobic interactions between hydrophobic residues of the peptide and the lipid acyl chains. Pore formation mediates leakage of ions, blockage of amino acid uptake, and intracellular ATP depletion (Ennahar *et al.*, 2000; Héchard and Sahl, 2002). ATP depletion may be a result of accelerated consumption of ATP to maintain and restore PMF and/or the inability to produce ATP as a result of inorganic phosphate efflux (Ennahar *et al.*, 2000). Another model used is a 'carpet-like' model (Fig. 3B), explaining the peptide-induced pore formation, where a single peptide molecule collapses as a result of a strong phospholipid mobilising activity, resulting in local and transient permeability (Moll *et al.*, 1999).

Structural features, including the β-turns, α-helixes, YGNGV motifs, and disulphide bridges of a bacteriocin, play one or more roles in the recognition and activity of the bacteriocin. The β-sheet exerts antimicrobial activity, while the α-helix is responsible for target specificity (Moll *et al.,* 1999). The YGNGV motif in the β-turn structure is responsible for the recognition step in the mechanisms of action. The YGNGV motif is easily exposed and recognised by a membrane receptor being positioned correctly. Another component responsible for recognition is the hydrophilic/amphiphilic N-termini of the β-sheet, together with its significant role in electrostatic membrane-bacteriocin interaction. Both the N-termini of the β-sheet and the YGNGV motif do not determine the specificity of activity. The central part of the α-helix regions are hydrophilic/slightly amphiphilic, and responsible for anchoring of a bacteriocin into the cytoplasmic membrane. The hydrophobic/amphiphilic C-terminal part plays an essential role in the insertion of a bacteriocin into the cytoplasmic membrane, the formation of a water-filled pore, and the major part in target-cell specificity of the bacteriocin. It is expected that class IIa bacteriocins with similar C-terminal sequences will display similar spectra of activity. Another important feature in activation is the disulphide bridges, determining the range of activity. The more bridges a bacteriocin has, the broader and greater the spectrum of activity will be (Moll *et al.,* 1999; Ennahar *et al.,* 2000; Richard *et al*., 2006).

All class IIb bacteriocins depend on the activity of two distinct peptides for activity. These peptides dissipate the transmembrane potential, while only a few dissipate changes in pH gradient. Dissipation of the PMF is usually induced by interacting with a specific receptor molecule, resulting in the formation of an anionic- and cationic-specific pore in the membrane, causing permeability (Ennahar *et al.,* 2000; Héchard and Sahl, 2002). Class IIc bacteriocins are a heterogeneous group of miscellaneous peptides, using various different modes of action, resulting in membrane permeability, and specific inhibition of septum formation and pheromone activity (Ennahar *et al.,* 2000; Héchard and Sahl, 2002). Studies of various class II modes of action studies have been carried out, but results are still not fully understood.

3.5. Bacteriocin production

Enterococcal bacteriocins have gained interest because of their activity against food-borne pathogens. Enterococci isolated from food and other environments have been shown to produce bacteriocins, usually referred to as enterocins. Enterocins are usually characterised as class II bacteriocins exhibiting activity towards listeriae, but a few exceptions do occur (Giraffa, 1995; Floriano *et al.,* 1998; Franz *et al.,* 1999). The anti-*Listeria* activity may be explained by the fact that enterococci and listeriae are phylogenetically closely related (Franz *et al.,* 1999). Not all enterocins can be readily grouped into various bacteriocin classes. Enterocins are characterised into class I, class IIa, class IIb, class IIc and class III bacteriocins (see Table 4) (Franz *et al*., 1999; Franz and Holzapfel, 2004; Moreno *et al.,* 2005). Enterocins have been extensively studied, especially those produced by *E. faecalis* and *E. faecium*. Enterocin AS-48, produced by *E. faecalis* S-48, is the first enterocin purified to homogeneity (Galvez *et al.,* 1989; Martinez-Bueno *et al.,* 1994).

Table 4: Classification of enterocins

Enterocins	**Producer strain**	**Size (kDa)**	**Source**	**Reference**
Class I				
Cytolycin (*Cyl*L_L)	*E. faecalis* DS16	3.437		Gilmore *et al*. (1994)
Cytolycin (*Cyl*L_S)	*E. faecalis* DS16	2.031		Gilmore *et al.* (1994)
Class IIa				
Enterocin A	*E. faecium* CTC492	4.833	Dry-fermented sausages	Aymerich *et al.* (1996)
Enterocin CRL 35	*E. faecium* CRL 35	4.290	Regional Argentinean (Tafi) cheese	Farias *et al.* (1996)
Enterocin SE-K4	*E. faecalis* K-4	4.918	Grass silage in Thailand	Eguchi *et al.* (2001)

Table 4: (continued)

Enterocins	Producer strain	Size (kDa)	Source	Reference
Bacteriocin 31	*E. faecalis* Y1717	5.009	Clinical isolate	Tomita *et al.* (1996)
Bacteriocin N15	*E. faecium* N15	3.500	Japanese rice-bran paste	Losteinkit *et al.* (2001)
Bacteriocin RC714	*E. faecium* RC714	4.937	Human faecal sample	Del Campo *et al.* (2001)
Mundticin	*E. mundtii* AT06	4.290	Minimally processed vegetables	Bennik *et al.* (1998)
Mundticin KS	*E. mundtii* NFRI 7393	4.290	Grass silage in Thailand	Kawamoto *et al.* (2002)
Mundticin QU2	*E. mundtii* QU 2	4.290	Soybean	Zendo *et al.* (2005)
Class IIb				
Enterocin L50A	*E. faecium* L50	5.190	Spanish dry-fermented sausages	Cintas *et al.* (1998)
Enterocin L50B	*E. faecium* L50	5.178		
Enterocin 1071A	*E. faecalis* BEF 1071	4.286	Porcine faeces in Göttingen	Balla *et al.* (2000); Franz *et al.* (2002)
Enterocin 1071B	*E. faecalis* BEF 1071	3.899	Porcine faeces in Göttingen	Balla *et al.* (2000); Franz *et al.* (2002)
Enterocin A5-11A	*E. durans*	5206	Mongolian airag	Batdorj *et al.* (2006)
Enterocin A5-11B	*E. durans*	5218	Mongolian airag	Batdorj *et al.* (2006)
Class IIc				
Enterocin AS-48	*E. faecalis* S-48	7.167	Porcine GIT	Martinez-Bueno *et al.* (1994)
Enterocin B	*E. faecium* T136	5.465	Dry-fermented sausages	Casaus *et al.* (1997)
Enterocin P	*E. faecium* P13	4.630	Dry-fermented sausages	Cintas *et al.* (1997)
Enterocin EJ97	*E. faecalis* EJ97	5.328	Municipal water	Galvez *et al.* (1998); Sanchez-Hidalgo *et al.* (2003)
Enterocin Q	*E. faecium* L50	3.952	Spanish dry-fermented sausages	Cintas *et al.* (2000)
Enterocin RJ-11	*E. faecalis* RJ-11	5.049	Rice bran	Yamamoto *et al.* (2003)
Class III				
Enterolysin A	*E. faecalis* LMG 2333	34.501	LMG Panel	Nilsen *et al.* (2003)

In and between the different classes of enterocins, differences and similarities do occur. For instance, enterocin B and enterocin 1071 A and B, share class IIa characteristics, but do not contain the unique YGNGVXaaC consensus motif (Casaus *et al.,* 1997). Enterocins L50A and L50B are synthesised without a N-terminal leader sequence or signal peptide (Cintas *et al.,* 1998). Enterocin AS-48 is a cyclic peptide with a broad spectrum of activity against Gram-positive and Gram-negative bacteria and is therefore considered as a peptide antibiotic (Galvez *et al.,* 1989; Martinez-Bueno *et al.,* 1994).

In general, class I bacteriocins have a fairly broad spectrum of activity, inhibiting bacteria in the genera *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus,* and to a lesser extent *Clostridium, Staphylococcus,* and *Bacillus* (Franz and Holzapfel, 2004). Class II bacteriocins have a narrow spectra of activity, normally inhibiting members of the genera *Enterococcus, Lactobacillus* and *Pediococcus,* with, in some cases, activity against *Clostridium, Bacillus* and in class IIa *Listeria* (Chen and Hoover, 2003; Franz and Holzapfel, 2004). Antiviral activity has been observed for enterocin CRL35 and ST4V, by activity against thymidine-kinase positive and deficient strains of herpes simplex types 1 and 2 in Vero and BHK-21 cells, affecting intracellular viral multiplication, and inhibiting late stages of replication (Wachsman *et al.,* 1999; Wachsman *et al.,* 2003; Todorov *et al.,* 2005). According to Wachsman *et al.* (2003), enterocin CRL35 did not inhibit the viruses in a direct manner, but affected protein synthesis (γ-protein synthesis), without altering virus uptake. Besides, enterocin CRL35 inhibited virus-induced cell fusion and virus spread without toxic effects to the host. The spread was affected by the accumulation of progeny viruses in the cell (Wachsman *et al*., 2003).

3.6. Enterococci as starter cultures and probiotics

Bacteriocin-producing enterococci may be exploited as commercial starter cultures and have been used for decades as a probiotic in the promotion of human and animal health (Lewenstein *et al.*, 1979; Bellomo *et al.*, 1980; Underdahl, 1983; Giraffa *et al.*, 1997).

One of the best-studied enterococcal probiotics is *E. faecium* SF68. *E. faecium* SF68 is commensal in the intestine, has a short lag phase and generation time, with moderate resistance to antibiotics and low pH. *E. faecium* SF68 is insensitive to bile salts, which makes it effective for treatment (Lewenstein *et al.*, 1979; Bellomo *et al.*, 1980). The probiotic is used as an alternative for antibiotic treatment of diarrhoea and shortens the period by one to three days. SF68 is effective for both children and adults, by decreasing the duration of diarrhoeal symptoms and stool normalisation time in patients. *E. faecium* SF68 is inhibitory against strains of *Escherichia coli, Shigella, Salmonella* species, and *Enterobacter* species. SF68 has also been used in animals to increase lactase concentrations in the small intestine, stimulating LAB growth and repressing the growth of intruding pathogens (Lewenstein *et al.,* 1979; Bellomo *et al.,* 1980; Moreno *et al.,* 2005). *E. faecium* SF68, used in dry dog food, enhanced specific immune functions, such as cell-mediated and humoral immune functions (Benyacoub *et al.,* 2003). In fermented milk products, *E. faecium* SF68 causes a hypocholesterolemic effect on individuals (Agerback *et al.,* 1995).

Causidofi is a probiotic consisting of two *Streptococcus thermophilus* strains and one strain of *E. faecium*. The probiotic is claimed to be hypocholesterolaemic in the short-term (Agerholm-Larsen *et al.*, 2000). No signs of long-term reduction of LDL-cholesterol are demonstrated, but studies indicate uncertainty of this effect in clinical relevance (Richelsen *et al.,* 1996; Lund *et al.,* 2002).

Bacterial bile salt hydrolase (BSH) activity is linked to the reduction of cholesterol. Various Gram-positive bacteria, including *Enterococcus* species, have BSH activity (Franz and Holzapfel, 2004). Marteau *et al.* (1995) suggest that BHS activity is common among enterococci, with 81% occurrence in *E. faecalis*, 50% in *E. faecium,* and 44% in *E. durans.*

More enterococcal strains with potential probiotic properties are *E. faecium* CRL 183 and *E. faecium* PR88, with decreasing cholesterol levels *in vitro* in combination of *Lactobacillus jugurti* and alleviation of symptoms of irritable bowl syndrome in humans, respectively (Allen *et al.,* 1996; Rossi *et al.,* 1999). Walthers ECOFLOR is an *E. faecium*-containing probiotic, with anticarcinogenic effects, efficient against diarrhoea, active against *L. monocytogenes*, with possible reduction of LDL-cholesterol levels, and is sensitive to vancomycin and L (+)-lactic acid production (Moreno *et al.,* 2005).

The use of enterococci as starter or co-cultures has increased considerably. Enterococci have the ability to withstand pasteurising temperatures, and to adapt to different substrates and growth conditions. This makes them good candidates to be found in food products manufactured from raw materials (milk or meat) and heat-treated food products. Enterococci contribute to the organoleptic properties of fermented food products and by producing enterocins, and are therefore important in food technology (Herranz *et al.,* 2001; Franz and Holzapfel, 2004). Various studies support the ability of enterococcal strains to inhibit spoilage organisms *Listeria* and *Clostridia*, as well as Gram-negative *Escherichia coli* and *Vibrio cholera* (Galvez *et al.,* 1989; Simonetta *et al.,* 1997; Moreno *et al.,* 2005). Enterococci, mainly *E. faecalis, E. faecium* and *E. durans*, are used as starter cultures in cheese and, in some cases, in meat products. The strains are included in various European cheeses, such as Cheddar, Feta, water-buffalo Mozzarella, Venaco, Cebreiro, Hispanico, and many more. Enterococcal cheese-starter cultures consist of various abilities, such as acidifying activity (important in any fermentation), proteolytic activity (breakdown of milk casein in ripening of cheeses), lipolytic activity (breakdown of substrates in solution, and texture), esterase activity (breakdown of substrates in solution, and texture), and citrate metabolism (formation of products and metabolites other than lactic acid). Some metabolic end products play a role in

aromatic properties such as diacetyl, acetaldehyde, acetoin, texture (CO_2), flavour, and colour (Franz and Holzapfel, 2004; Moreno *et al.,* 2005).

The increasing emergence of antibiotic resistance in enterococci, the presence of virulent determinants, and its association with human disease, makes the use of enterococci as probiotic still a controversial issue. To use enterococci as a probiotic, the antibiotic resistance and virulence determinants have to be determined (Moreno *et al.,* 2005).

4. References

Abee, T., L. Krockel, and C. Hill. 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. Int. J. Food Microbiol. 28, 169-185.

Agerback, M., L.U. Gerdes, and B. Richelsen. 1995. Hypocholesterolaemic effect of a new fermented milk product in healthy middle-aged men. Eur. J. Clin. Nut. 49, 346-352.

Agerholm-Larsen, L., M.L. Bell, G.K. Grunwald, and A. Astrup. 2000. The effect of milk probiotic on plasma cholesterol: a meta-analysis of short-term intervention studies. Eur. J. Clin. Nutr. 54(11), 856-860.

Agro, A.S. 1999. Meeting the challenge of chronic suppurative otitis media. Infect. Dis. Infoalert, 1-9.

Aimoni, C., S. Pelucchi, D.L. Grasso, M. Libanore, and A. Martini. 2005. Bacterial meningitis complicating suppurative otitis media and sinusitis. Otolaryngol. Head Neck Surg. 132(6), 965-966.

Allen, W.D., M.A. Linggood, and P. Porter. 1996. *Enterococcus* organisms and their use as probiotics in alleviating irritable bowel syndrome symptoms. European Patent 0508701 (B1).

Alonsodevelasco, E., A.F.M. Verheul, J. Verhoef, and H. Snippe. 1995. *Streptococcus pneumoniae:* Virulence factors, pathogenesis, and vaccines. Microbiol. Rev. 59(4), 591-603.

American Academy of Family Physicians. American Academy of Otolaryngology–Head and Neck Surgery, American Academy of Pediatrics. Subcommitee on Otitis Media with effusion. 2004. Otitis media with effusion. Pediatr. 113(5), 1412-1429.

American Academy of Pediatrics and American Academy of Family Physicians, Clinical Practice Guidelines. Subcommittee on Management of Acute Otitis Media. 2004. Diagnosis and management of acute otitis media. Pediatr. 133(5), 1451-1465.

Andrews, F.W., and T.J. Horder. 1906. A study of the streptococci pathogenic for man. Lancet 2, 708-713.

Appelbaum, P.C. 1992. Antimicrobial resistance in *Streptococcus pneumoniae*. Clin. Infect. Dis. 15, 77-83.

Appelbaum, P.C. 1995. New prospects for antibacterial agents against multidrug-resistant pneumococci. Microb. Drug Resist. 1, 43-48.

Archimbaud, C., N. Shankar, C. Forestier, A. Baghdayan, M.S. Gilmore, F. Charbonne, and B. Joly. 2002. *In vitro* adhesive properties and virulence factors of *Enterococcus faecalis* strains. Res. Microbiol. 153, 75-80.

Augustsson, I., and I. Engstand. 2001. Otitis media and academic achievements. Int. J. Pediatr. Otorhinolaryngol. 57, 31-40.

Axelson, L. 2004. Lactic acid bacteria: Classification and Physiology. In: Salminen, S., von Wright, A. and Ouwenhand, A. (Eds.). Lactic acid bacteria: microbiological and functional aspects, 3rd ed. Marcel Dekker Inc., New York. pp. 1-61.

Aymerich, T., H. Holo, L.S. Håvarstein, M. Hugas, M. Garriga, and I.F. Nes. 1996. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium,* a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl. Environ. Microbiol. 62, 1676-1682.

Baele, M., P. Baele, M. Vaneechoutte, V. Storms, P. Butaye, L.A. Devriese, G. Verschraegen, M. Giklis, and F. Haesebrouck. 2000. Application of tRNA ingenic spacer PCR for identification of *Enterococcus* species. J. Clin. Microbiol. 38, 4201-4207.

Balla, E., L.M.T. Dicks, M. du Toit, M.J. van der Merwe, and W.H. Holzapfel. 2000. Characterization and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by *Enterococcus faecalis* BFE 1071. Appl. Environ. Microbiol. 66, 1298-1304.

Bamberger, D.M., and M.A. Jackson. 2001. Upper respiratory tract infections: pharyngitis, sinusitis, otitis media, and epiglottitis. In: Niederman, M.S., Sarosi, G. A. and Glassroth, J. (Eds.). Respiratory Infections, 2nd ed. Lippincott Williams and Wilkens, Philadelphia. pp. 125-139.

Barden, L.S., S.F. Dowell, B. Schwartz, and C. Lackey. 1998. Current attitudes regarding use of antimicrobial agents: results from physicians' and parents' focus group discussions. Clin. Pediatr. (Phila.) 37, 665-671.

Batdorj, B., M. Dalgalarrondo, Y. Choiset, J. Pedroche, F. Metro, H. Prevost, J.M. Chobert, and T. Haertle. 2006. Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. J. Appl. Microbiol. 101(4):837-848.

Bellomo, G., A. Mangiagle, L. Nicastro, and G. Frigerio. 1980. A controlled double-blind study of SF68 strain as a new biological preparation for the treatment of diarrhoea in paediatrics. Curr. Ther. Res. Clin. Exp. 28, 927-934.

Bennik, M.H.J., B. Vanloo, R. Brasseur, L.G.M. Gorris, and E.J. Smid. 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: Full charaterization and interaction with target organism. Biochemica et Biophysica Acta 1373, 47-58.

Benyacoub, J., G.L. Czarnecki-Maulden, C. Sauthier, R.E. Anderson, E.J. Schiffrin, and T. von der Weid. 2003. Supplementation of food with *Enterococcus faecium* (SF68) stimulates immune functions in young dogs. J. Nutr. 133, 1158-1162.

Bernstein, J.M., H.F. Faden, D.M. Dryja, and J. Wactawski-Wende. 1993. Micro-ecology of the nasopharyngeal bacterial flora in otitis-prone and non-otitis-prone children. Acta Otolaryngol. 113, 88-92.

Bernstein, J.M., S. Sagahtaheri-Altaie, D.M. Dryjd, and J. Wactawski-Wende. 1994. Bacterial interference in nasopharyngeal bacterial flora of otitis-prone and non-otitis-prone children. Acta oto-laryngologica, Belgium, 48, 1-9.

Block, S.L. 1997. Causative pathogens, antibiotic resistance and therapeutic considerations in acute otitis media. Pediatr. Infect. Dis. J. 16(4), 449-456.

Blomgren, K. and A. Pitkäranta. 2003. Is it possible to diagnose acute otitis media accurately in primary health care? Fam. Pract. 20(5), 524-527.

Blomgren, K. and A. Pitkäranta. 2005. Current challenges in diagnosis of acute otitis media. Int. J. Pediatr. Otorhinolaryngol. 69, 295-299.

Booth, M.C., C.P. Bogie, H-G., Sahl, R.J. Siezen, K.L. Hatter, and M.S. Gilmore. 1996. Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolycin, a novel antibiotic. Mol. Microbiol. 21, 1175-1184.

Brogden, K.A. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nature Rev. Microbiol. 3, 238-250.

3:1-13, 2005.

Brook, I. 2003. Effects of antimicrobial therapy on the microbial flora of the adenoids. J. Antimicrob. Chemoth. 51, 1331-1337.

Brook, I. 2005. The role of bacterial interference in otitis, sinusitis, and tonsillitis. Otolaryngol. Head Neck Surg. 133, 139-146.

Brook, I., and P.A. Foote. 1997. Bacterial interference and beta-lactamase-producing bacteria in the adenoids after antimicrobial therapy. Rev. Infect. Dis. 25, 493.

Brook, I, and A.E. Gober. 1998. Bacterial interference in the nasopharynx following antimicrobial therapy of acute otitis media. J. Antimicrob. Chemoth. 41, 489-492.

Brook, I., and A.E. Gober. 2000. *In vitro* bacterial interference in the nasopharynx of otitis media-prone and non-otitis media-prone children. Arch. Otolaryngol. Head Neck Surg. 126, 1011-1013.

Brook, I., and A.E. Gober. 2005a. Antimicrobial resistance in the nasopharyngeal flora of children with acute otitis media and otitis media recurring after amoxicillin therapy. J. Med. Microbiol. 54, 83-85.

Brook, I., and A.E. Gober. 2005b. Long-term effects on the nasopharyngeal flora of children following antimicrobial therapy of acute otitis media with cefdinir or amoxicillin-clavulanate. J. Med. Microbiol. 54, 553-556.

Brook, I., and P. Yocum. 1999. Bacterial interference in the adenoids of otitis media-prone children. Pediatr. Infect. Dis. J. 18, 835-837.

Buchman, C.A., and G.M. Brinson. 2003. Viral otitis media. Curr. Allergy Asthma Rep. 3(4), 335-340.

Casaus, P., T. Nilsen, L.M. Cintas, I.F. Nes, P.E. Hernandez, and H. Holo. 1997. Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. Microbiol. 143, 2287-2294.

Casey, J.R, and M.E. Pichichero. 2004. Changes in frequency and pathogens causing acute otitis media in 1995-2003. Pediatr. Infect. Dis. J. 23(9), 824-828.

Cayé-Thomasen, P., and M. Tos. 2004. Eustachian tube gland tissue changes are related to bacterial species in acute otitis media. Int. J. Pediatr. Otolaryngol. 68, 101-110.

Chen, H. and D.G. Hoover. 2003. Bacteriocins and their food applications. Compr. Rev. Food. Science Food Safety 2, 82-100.

Chenoweth, C., and D. Schaberg. 1990. The epidemiology of enterococci. Eur. J. Clin. Microbiol. Infect. Dis. 9, 80-89.

Chow, J.W., L.A. Thai, M.B. Perri, J.A. Vazquez, S.M. Donabedjan, D.B. Clewel, and M.J. Zervos. 1993. Plasmid-associated hemolycin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. Antimicrob. Agents Chemother. 37, 2474-2477.

Cintas, L.M., P. Casaus, L.S. Håvarstein, P.E. Hernández, and I.F. Nes. 1997. Biochemical and genetic characterization of enterocin P, a novel *sec*-dependent bacteriocin

from *Enterococcus Faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. 63, 4321-4330.

Cintas, L.M., P. Casaus, C. Herranz, L.S. Håvarstein, H. Holo, P. Hernandez, and I.F. Nes. 2000. Biochemical and genetic evidence that *Enterococcus faecium* L50 produces enterocins L50A and L50B, *sec*-dependent enterocin P, and a novel bacteriocin secreted without a N-terminal extension termed enterocin Q. J. Bacteriol. 182, 6806-6814.

Cintas, L.M., P. Cassaus, H. Holo, P.E. Hernandez, I.F. Nes, and L.S. Havarstein. 1998. Enterocins L50A and L50B, two novel bacteriocins from *E. faecium* L50, are related to staphylococcal hemolysins. J. Bacteriol. 180, 1988-1994.

Cleveland, J., T.J. Montville, I.F. Nes, and M.L. Chikindas. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. 71, 1-20.

Clewell, D.B. 1990. Movable genetic elements and antibiotic resistance in enterococci. Eur. J. Clin. Microbiol. Infect. Dis. 9, 90-102.

Clewell, D.B., M.V. Francia, and S.E. Flannagan. 2002. Enterococcal plasmid transfer: sex pheromones, transfer origins, relaxases, and the *Staphylococcus aureus* issue. Plasmids 48, 193-201.

Clewell, D.B., Y. Yagi, G.M. Dunny, and S.K. Schulz. 1974. Characterization of three plasmids deoxyribonucleic acid molecules in a strain of *Streptococcus faecalis:* identification of a plasmid determining erythromycin resistance. J. Bacteriol. 117, 283-289.

Cripps, A.W., D.C. Otczyk, and J.M. Kyd. 2005. Bacterial otitis media: a vaccine preventable disease? Vaccine 23, 2304-2310.

Dagan, R., and E. Leibovitz. 2002. Bacterial eradication in the treatment of otitis media. Lancet Infect. Dis. 2, 593-604.

Dagan, R., N. Givon-Lavi, O. Zamir, and D. Fraser. 2003. Effect of a 9-valent conjugate vaccine on carriage of antibiotic-resistant *Streptococcus pneumoniae* in day-care centers. Pediatr. Infect. Dis. J. 22, 532-539.

Dagan, R., M. Sikuler-Cohen, O. Zamir, J. Janco, N. Givon-Lavi, and D. Fraser. 2001. Effect of a conjugate pneumococcal vaccine on the occurrence of respiratory infections and antibiotic use in day-care center attendees. Pediatr. Infect. Dis. J. 20, 951-958.

Deasy, B.M., M.C. Rea, G.F. Fitzgerald, T.M. Cogan, and T.P. Beresford. 2000. A rapid PCR method to distinguish between *Lactococcus* and *Enterococcus*. Syst. Appl. Microbiol. 23, 510-522.

de Gutierrez, R.C., V. Santos, and M.E. Nader-Macias. 2001. Protective effect of intranasally inoculated *Lactobacillus fermentum* against *Streptococcus pneumoniae* challenge on the mouse respiratory tract. FEMS Immunol. Med. Microbiol. 31, 187-195.

del Campo, R., C. Tenorio, R. Jiménez-Diaz, C. Rubio, R. Gomez-Lus, F. Baquero, and C. Torres. 2001. Bacteriocin production in vancomycin-susceptible *Enterococcus* isolates of different origin. Antimicrob. Agents Chemoch. 45, 905-912.

de Ru, J.A., and J.J. Grote. 2004. Otitis media with effusion: disease or defence? Int. J. Pediatr. Otorhinolaryngol. 68, 331-339.

Devriese, L.A., and B. Pot. 1995. The genus *Enterococcus*. In: Wood, B.J.B. and Holzapfel, W.H. (Eds.). The Genera of Lactic Acid Bacteria. Blackie Academic and Professional, London. pp. 327-367.

Devriese, L.A., B. Pot, and M.D. Collins. 1993. Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. J. Appl. Bacteriol. 75, 399-408.

De Vuyst, L., and E.J. Vandamme. 1994. Antimicrobial potential of lactic acid bacteria. In: De Vuyst, L., and Vandamme, E.J. (Eds.). Bacteriocins of lactic acid bacteria: Microbiology, Genetics and Application. Blackie Academic and Professional, London. pp. 91-142.

Dierksen, K. and J. Tagg. 2000. The influence of indigenous bacteriocin-producing *Streptococcus salivarius* on the acquisition of *Streptococcus pyogenes* by primary school children in Dunedin, New Zealand. In: Martin, D. and Tagg, J. (Eds.). Streptococci and Streptococcal Disease entering the New Millennium. Securacopy, Wellington, New Zealand. pp. 81-85.

Doi, K., T. Eguchi, A. Twatake; J. Shima, S. Ohmomo, and S. Ogata. 2002. Isolation of enterocin SE_K4-encoding plasmid and a high enterocin SE-K4 producing strain of *Enterococcus faecalis* K-4. J. Biosci. Bioeng. 93(4), 434-436.

Dowell, S.F., J.C. Butler, G.S. Giebink, M.R. Jacobs, D. Jernigan, D. Musher, A. Rakowsky, and B. Schwartz. 1999. Acute otitis media: management and surveillance in an era of pneumococcal resistance - a report from the drug-resistant *Streptococcus pneumoniae* therapeutic working group. Pediatr. Infect. Dis. J. 18, 1-9.

Dupont, H., P. Montravers, J. Mohler, and C. Carbon. 1998. Disparate findings on the role of virulence factors of *Enterococcus faecalis* in mouse and rat models of peritonitis. Infect. Immun. 66, 2570-2575.

Eaton, T.J., and M.J. Gasson. 2002. A variant enterococcal surface protein Esp_{fm} in *Enterococcus faecium*; distribution among food, commensal, medical, and environmental isolates. FEMS Microbiol. Lett. 216, 269-275.

Eguchi, T., K. Kaminaka, J. Shima, S. Kawamoto, K. Mori, S-H. Choi, K. Doi, S. Ohmomo, and S. Ogata. 2001. Isolation and characterization of enterocin SE-K4 produced by thermophilic enterococci, *Enterococcus faecalis* K4. Biosci. Biotechnol. Biochem. 65, 247-253.

Eijsink, V.G., M. Skeie, P.H. Middelhoven, M.B. Brurberg, and I.F. Nes. 1998. Comparative studies of class 2a bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. 64, 3275-3281.

Endz, H.P., N. van den Braak, H.A. Verbrugh, and A. van Belkum. 1999. Vancomycin resistance: status quo and quo vadis. Eur. J. Clin. Microbiol. Infect. Dis. 18, 683-690.

Eneli, I.U. 1998. Otitis Media: An Update. Medical Update for Psychiatrist 3(5), 165-169.

Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. 24, 85-106.

Eskola, J., and T. Kilpi. 1999. Efficacy of a heptavalent pneumococcal conjugate vaccine (PncCRM) against serotype-specific, culture-confirmed pneumococcal acute otitis media in infants and children. Presented at: 39th Interscience Conference on Antimicrobial Agents and Chemotherapy; September 26-29, San Francisco, CA.

Eskola, J., T. Kilpi, A. Palmu, J. Jokinen, J. Haapakoski, E. Herva, A. Takala, H. Käyhty, P. Karma, R. Kohberger, G. Siber, and P.H. Mäkelä. 2001. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N. Engl. J. Med. 344, 403-409.

Faden, H., L. Duffy, and R. Wasielewski. 1997. Relationship between nasopharyngeal colonization and the development of otitis media in children. J. Infect. Dis. 175, 1440-1445.

Fainstein, V., and D.M. Musher. 1979. Bacterial adherence to pharyngeal cells in smokers, non-smokers, and chronic bronchitis. Infect. Immun. 26, 178-182.

Farias, M.E., R.N. Farias, A.P. de Ruiz Holgado, and F. Sesma. 1996. Purification and N-terminal amino acid sequence of enterocin CRL 35, a 'pediocin-like' bacteriocin produced by *Enterococcus faecium* CRL 35. Lett. Appl. Microbiol. 22, 417-419.

Fendrick, A.M., S. Saint, I. Brook, M.R. Jacobs, S. Pelton, and S. Sethi. 2001. Diagnosis and treatment of upper respiratory tract infections in the primary care setting. Clin. Ther. 23(10), 1683-1706.

Fireman, P. 1997. Otitis media and eustachian tube dysfunction: connection to allergic rhinitis. J. Allergy Clin. Immunol. 99, S787-S797.

Fireman, B., S. Black, H. Shinefield, J. Lee, E. Lewis, and P. Ray. 2003. Impact of the pneumococcal conjugate vaccine on otitis media. Pediatr. Infect. Dis. J. 22, 10-16.

Floriano, B., J.L. Ruiz-Barba, and R. Jiménez-Diaz. 1998. Purification and genetic characterization of enterocin I from *Enterococcus faecium* 6T1a, a novel antilisterial plasmid-encoded bacteriocin which does not belong to the pediocin family of bacteriocins. Appl. Environ. Microbiol. 64(12), 4883-4890.

Fluit, A.C., M.R. Visser, and F-J. Schmitz. 2001. Molecular detection of antimicrobial resistance. Clin. Microbiol. Rev. 14(4), 836-871.

Franke, A.E., and D.B. Clewell. 1981. Evidence for a chromosome-borne resistance transposon (Tn916) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. 145, 494-502.

Franz, C.M.A.P., and W.H. Holzapfel. 2004. The Genus Enterococcus: biotechnological and safety issues. In: Salminen, S. A. von Wright, A and Ouwenhand A. (Eds.). Lactic acid bacteria: microbiological and functional aspects, 3rd ed. Marcel Dekker Inc., New York. pp. 199-247.

Franz, C.M.A.P., A. Grube, A. Hermann, H. Abriouel, J. Stärke, A. Lombardi, B. Tauscher, and W.H. Holzapfel. 2002. Biochemical and genetic characterization of the two-peptide bacteriocin enterocin 1071 produced by *Enterococcus faecalis* FAIR-E 309. Appl. Environ. Microbiol. 68, 2550-2554.

Franz, C.M.A.P., W.H. Holzapfel, and M.E. Stiles. 1999. Enterococci at the crossroads of food safety? Int. J. Food Microbiol. 47, 1-24.

Fujimori, I., K. Hisamatsu, K. Kikushima, R. Goto, Y. Murakami, and T. Yamada. 1996. The nasopharyngeal bacterial flora in children with otitis media with effusion. Eur. Arch. Otorhinolaryngol. 253(4-5), 260-263.

Galvez, A., M. Maqueda, M. Martinez-Bueno, and E. Valdivia. 1989. Bactericidal and bacteriolytic action of peptide antibiotic AS-48 against gram-positive and gram-negative bacteria and other organisms. Res. Microbiol. 140, 57-68.

Galvez, A., E. Valdivia, H. Abriouel, E. Camafeita, E. Mendez, M. Martinez-Bueno, and M. Maqueda. 1998. Isolation and characterization of enterocin EJ97, a bacteriocin produced by *Enterococcus faecalis* EJ97. Archives Microbiol. 171, 59-65.

Gasson, M.J. 1990. *In vivo* genetic systems in lactic acid bacteria. FEMS Microbiol. Rev. 7, 43-60.

Gibson, E.M., N.M. Chace, S.B. London, and J. London. 1979. Transfer of plasmid-mediated antibiotic resistance from streptococci to lactobacilli. J. Bacteriol. 137, 614-619.

Gilmore, M.S., R.A. Segarra, M.C. Booth, Ch.P. Bogie, L.R. Hall, and D.B. Clewell. 1994. Genetic structure of *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. J. Bacteriol. 176, 7335-7344.

Giraffa, G. 1995. Enterococcal bacteriocins: their potential as anti-*Listeria* factors in dairy technology. Food Microbiol. 12, 291-299.

Giraffa, G., D. Carminati, and E. Neviani. 1997. Enterococci isolated from diary products: a review of risks and potential technological use. J. Food Protection 60, 732-738.

Gonzalez, C.F., and B.S. Kunka. 1983. Plasmid transfer in *Pediococcus* spp: intergeneric and intrageneric transfer of pIP501. Appl. Environ. Microbiol. 46, 81-89.

Gould, I.M. 1998. Antibiotic policies and control of resistance. Curr. Opin. Infect. Dis.15, 395-400.

Guidelines and Protocols Advisory Committee. 2004. Otitis media with effusion. Pediatrics 113, 1412-1428.

Guzman, C.A., C. Pruzzo, and L. Calegari. 1991b. *Enterococcus faecalis*: specific and non-specific interactions with human polymorphonuclear leukocytes. FEMS Microbiol. Lett. 68, 157-162.

Guzman, C.A., C. Pruzzo, G. LiPira, and L. Calegari. 1989. Role of adherence in pathogenesis of *Enterococcus faecalis* urinary tract infection and endocarditis. Infect. Immun. 57, 1834-1838.

Guzman, C.A., C. Pruzzo, M. Plate, M.C. Guardati, and L. Calegari. 1991a. Serum dependent expression of *Enterococcus faecalis* adhesions involved in the colonization of heart cells. Microb. Pathog. 11, 399-409.

Hansman, D., and M.M. Bullen. 1967. A resistant pneumococcus (Letter). Lancet 2, 264-265.

Hastie, A.T., K.B. Everts, J. Zangrilli, J.R. Shaver, M.B. Pollice, J.E. Fish, and S.P. Peters. 1997. HSP27 elevated in mild allergic inflammation protects airway epithelium from H_2SO_4 effects. Am. J. Physiol. 273, L401-L409.

Håvarstein, L.S., D.B. Diep, and I.F. Nes. 1995. A family of bacteriocin ABC-transporters carry out proteolytic processing of their substrates concomitant with export. Mol. Microbiol. 16(2), 229-240.

Héchard, Y., and H-G. Sahl. 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie 84, 545-557.

Heikkinen, T, and T. Chonmaitree. 2003. Importance of respiratory viruses in acute otitis media. Clin. Microbiol. Rev. 16(2), 230-241.

Heikkinen, T, M. Thint, and T. Chonmaitree. 1999. Prevalence of various respiratory viruses in the middle ear during acute otitis media. N. Engl. J. Med. 340(4), 260-264.

Herranz, C., P. Casaus, S. Mukhopadhyay, J.M. Martinez, J.M. Rodrigues, I.F. Nes, P.E. Hernandez, and L.M. Cintas. 2001. *Enterococcus faecium* P21: a strain occuring naturally in dry-fermented sausages producing the class 2 bacteriocins enterocin A and enterocin B. Food Microbiol. 18, 115-131.

Holm, V.A., and L.V.K. Kunze. 1963. Effect of chronic otitis media on language and speech development. Pediatrics 43, 833-839.

Holt, J.G. 1994. Bergey's manual of determinative bacteriology. Williams and Wilkens, Baltimore.

Homoe, P., R.B. Christensen, and P. Bretlau. 2005. Acute otitis media and season of birth. Int. J. Pediatr. Otorhinolaryngol. 69, 487-491.

Horodniceanu, T., D.H. Bouanchaud, G. Bieth, and Y.A. Chabbert. 1976. R-plasmids in *Streptococcus agalactiae* (group B). Antimicrob. Agents Chemother. 10, 795-801.

Huebner, R.E., A.D. Wasas, and K.P. Klugman. 2000. Trends in antimicrobial resistance and serotyoe distribution of blood and cerebrospinal fluid isolates of *Streptotcoccus pneumoniae* in South Africa, 1991-1998. Int. J. Infect. Dis. 4(4), 214-218.

Huycke, M.M., W. Joyce, and M.F. Wack. 1996. Augmented production of extracellular superoxide by blood isolates of *Enterococcus faecalis*. J. Infect. Dis. 173(3), 743-746.

Jacobs, M.R.J. 2004. *Streptococcus pneumoniae:* Epidemiology and patterns of resistance. Am. J. Med. 117(3A), 3S-15S.

Jacobs, M.R.J., S. Bajaksouzian, A. Windau, C.E. Good, G. Lin, G.A. Pankuck, and P.C. Appelbaum. 1999. Susceptibility of *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis* to oral agents: results of a 1998 US outpatient surveillance study. Presented at: 39th Interscience Conference on Antimicrobial Agents and Chemotherapy; September 26-29, San Francisco, CA.

Jett, B.D., M.M. Huycke, and M.S. Gilmore. 1994. Virulence of enterococci. Clin. Microbiol. Rev. 7, 462-478.

Johnson, A.P. 1994. The pathogenicity of enterococci. J. Antimicrob. Chemother. 33, 1083-1089.

Jones, M.E., R.S. Blosser-Middleton, C. Thornsberry, J.A. Karlowsky, and D.F. Sahm. 2003. The activity of levofloxacin and other antimicrobials against clinical isolates of *Streptococcus pneumoniae* collected worldwide during 1999-2002. Diagn. Microbiol. Infect. Dis. 47, 579-586.

Kaieda, S., H. Yano, N. Okitsu, Y. Hosaka, R. Okamoto, M. Inoue, and H. Takahashi. 2005. Investigation about the homogeneity of nasopharyngeal microflora at the different location of nasopharynx of children with acute otitis media. Int. J. Pediatr. Otorhinolaryngol. 69, 959-963.

Kawamoto, S., J. Shima, R. Sato, T. Educhi, S. Ohmomo, J. Shibato, N. Horikashi, K. Takeshita, and T. Sameshima. 2002. Biochemical and genetic characterization of mundticin KS, an antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. Appl. Environ. Microbiol. 68, 3830-3840.

Kawano, H., A. Haruta, Y. Tsuboi, Y. Kim, P.A. Schchern, M.M. Paparella, and J. Lin. 2002. Induction of mucus cell metaplasia by tumor necrosis factor alpha in rat middle

ear: the pathological basis for mucin hyperproduction in mucoid otitis media. Ann. Otol. Rhinol. Laryngol. 111, 415-422.

Klaenhammer, T.R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12, 39-86.

Kleemola, M., J. Nokso-Koivisto, E. Herva; R. Syrjänen, M. Lahdenkari, T. Kilpi, and T. Hovi. 2006. Is there any specific association between respiratory viruses and bacteria in acute otitis media of young children? J. Infect. 52(3), 181-187.

Klein, J.O. 1989. Epidemiology of otitis media. Pediatr. Infect. Dis. 8(1), 89.

Klein, J.O. 1999. Management of acute otitis media in an era of an increasing antibiotic resistance. Int. J. Pediatr. Otorhinolaryngol. 49(1), S15-17.

Klugman, K.P., and H.J. Koornhof. 1988. Drug resistance patterns and serotypes of pneumococcal isolates from cerebrospinal fluid or blood, 1979-1986. J. Infect. Dis.158, 956-964.

Koornhof, H.J., A. Wasas, and K. Klugman. 1992. Antimicrobial resistance in *Streptococcus pneumoniae*: a South Africa perspective. Clin. Infect. Dis. 15, 84-94.

Kouwen, H., F.A.M. van Balen, and P.H. Dejonkere. 2005. Functional tube therapy for persistent otitis media with effusion in children: myth or evidence? Int. J. Pediatr. Otorhinolaryngol. 69, 943-951.

Kvaerner, K.J., K. Tambs, J.R. Harris, I.W. Mair, and P. Magnus. 1996. Otitis media: relationship to tonsillitis, sinusitis, and atopic diseases. Int. J. Pediatr. Otorhinolaryngol. 35, 127-141.

Leclercq, R. 1997. Enterococci acquire new kinds of resistance. Clin. Infect. Dis. 24(suppl.1), S80-S84.

Leclercq, R., S. Dutka-Malen, A. Brissom-Noël, C. Mas, E. Derlot, M. Arthur, J. Duval, and P. Courvalin. 1992. Resistance of Enterococcus to aminoglycosides and glycopeptides. Clin. Infect. Dis. 15, 495-501.

Lee, D.H., Y.S. Park, T.T. Jung, S.W. Yeo, Y.C. Choi, and E. Jeon. 2001. Effect of tumor necrosis factor-alpha on experimental otitis media with effusion. Laryngoscope 111, 728-733.

Leibovitz, E., and R. Dagan. 2000. Antibiotic treatment for acute otitis media. Int. J. Antimicrob. Agents 15, 169-177.

Leibovitz, E., and R. Dagan. 2001a. Otitis media therapy and drug resistance. Part 1: management principles. Infect. Med. 18, 212-216.

Leibovitz, E., and R. Dagan. 2001b. Otitis media therapy and drug resistance. Part 2: current concepts and new directions. Infect. Med. 18, 263-270.

Lewenstein, A., G. Frigerio, and M. Moroni. 1979. Biological properties of SF68, a new approach for the treatment of diarrhoeal diseases. Curr. Ther. Res. Clin. Exp. 26, 967-981.

Losteinkit C., K. Uchiyama, S. Ochi, T. Takaoka, K. Nagahisa, and S. Shioya. 2001. Characterization of bacteriocin N15 produced by *Enterococcus faecium* N15 and cloning of the related genes. Bioscience and Bioeng. 91(4), 390-395.

Loutonen, M., M. Uhari, L. Aitola, A.M. Lukkaroinen, J. Loutonen, and M. Uhari. 1998. A nation-wide, population-based survey of otitis media and school achievement. Int. J. Pediatr. Otorhinolaryngol. 43(1), 41-51.

Low, D.E. 2001. Mechanisms of antimicrobial resistance. In: Niederman, M.S., Sarosi, G.A. and Glassroth J. (Eds.). Respiratory infections, 2nd ed. Lippincott Williams and Wilkens, Philadelphia. pp. 93-107.

Lund, B., I. Adamsson, and C. Edlund. 2002. Gastrointestinal transit survival of an *Enterococcus faecium* probiotic strain administered with or without vancomycin. Int. J. Food Microbiol. 77, 109-115.

Lyon, J.L., A. Ashton, B. Turner, and M. Magill. 1998. Variation in the diagnosis of upper respiratory tract infections and otitis media in an urgent medical care practice. Arch. Fam. Med. 7(3), 249-254.

Marteau, P., M.F. Gerhardt, A. Myara, E. Bouvier, F. Trivin, and J.C. Rambaud. 1995. Metabolism of bile salt by alimentary bacteria during transit in the human small intestine. Microbial. Ecol. Health Dis. 8, 151-157.

Martinez-Bueno, M.M, M. Maqueda, A. Galvez, B. Samyn, J. van Beeumen, J. Coyette, and E. Valdivia. 1994. Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48. J. Bacteriol. 176, 6334-6339.

McCaig, L.F., and J.M. Hughes. 1995. Trends in antimicrobial prescribing among office-based physicians in the United States. JAMA 273(3), 214-219.

McCracken, G.H. 2002. Diagnosis and management of acute otitis media in the urgent care setting. Ann. Emerg. Med. 39, 413-421.

Moellering, Jr., R.C. 1992. Emergence of Enterococcus as a significant pathogen. Clin. Infect. Dis. 14, 1173-1178.

Moll, G.N., W.N. Konings, and A.J.M. Driessen. 1999. Bacteriocins: mechanism of membrane insertion and pore formation. Antonie van Leeuwenhoek 76, 185-198.

Montgomery, D. 2005. A new approach to treating acute otitis media. *J*. Pediatr. Health Care 19(1), 50-52.

Montravers, P., J. Mohler, L. Saint Julien, and C. Carbon. 1997. Evidence of the proinflammatory role of *Enterococcus faecalis* in polymicrobial peritonitis in rats. Infect. Immun. 65, 144-149.

Moreno, M.R.F., P. Sarantinopoulos, E. Tsakalidou, and L. de Vuyst. 2005. The role and application of enterococci in food and health. Int. J. Food Microbiol. 106(1), 1-24.

Morrison, D., N. Woodford, and B. Cookson. 1997. Enterococci as emerging pathogens of humans. J. Appl. Microbiol. Symp. Suppl. 83, 89S-99S.

Mundy, L.M., D.F. Sahm, and M. Gilmore. 2000. Relationship between enterococcal virulence and antimicrobial resistance. Clin. Microbiol. Rev. 13(4), 513-522.

Munoz, R.T., J. Coffey, M. Daniels, C. G. Dowson, G. Laible, J. Casal, R. Hakenbeck, M. Jacobs, J.M. Musser, and B.G. Spratt. 1991. Inter-continental spread of a multiresistant clone of serotype 23F *Streptococcus pneumoniae.* J. Infect. Dis. 164, 302-306.

Murphy, T.F., L.O. Bakeletz, J.M. Kyd, B. Watson, and D.L. Klein. 2005. Vaccines for otitis media: proposals for overcoming obstacles to progress. Vaccine 23, 2696-2702.

Murray, B.E. 1990. The life and times of *Enterococcus.* Clin. Microbiol. Rev. 3, 46-65.

Nes, I.F., B. D. Diep, L.S. Havarstein, M.B. Brurberg, V. Eijsink, and H. Holo. 1996. Biosynthesis of bacteriocins of lactic acid bacteria. Antonie van Leeuwenhoek 70, 113-128.

Nilsen, T., I.F. Nes, and H. Holo. 2003. Enterocycin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. Appl. Environ. Microbiol. 69, 2975-2984.

Nissen-Meyer, J., and I.F. Nes. 1997. Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. Archives Microbiol. 167, 67-77.

Noble, W.C., Z. Virani, and R.G. Cree. 1992. Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. FEMS Microbiol. Lett. 72, 195-198.

Nokso-Koivisto, J., R. Raty, S. Blomqvist, M. Kleemola, R. Syrjanen, A. Pitkaranta, T. Kilpi, and T. Hovi. 2004. Presence of specific viruses in the middle ear fluids and respiratory secretions of young children with acute otitis media. J. Med. Virol. 72(2), 241-248.

O'Keeffe, T., C. Hill, and R.P. Ross. 1999. Characterization and heterologous expression of the genes encoding enterocin A production, immunity, and regulation in *Enterococcus faecium* DPC1146. Appl. Environ. Microbiol. 65(4), 1506-1515.

Ozawa, Y., P. Courvalin, and M. Galimand. 2000. Identification of enterococci at the species level by sequencing of the genes for D-alanine:D-alanine ligases. Syst. Appl. Microbiol. 23, 230-237.

Paap, C.M. 1996. Management of otitis media with effusion in young children. Ann. Pharmacother. 30, 1291-1297.

Penido, N.D.O., A. Borin, LC.N. Iha, V.M. Suguri, E. Onishi, Y. Fukuda, and O.L.M. Cruz. 2005. Intracranial complications of otitis media: 15 years of experience in 33 patients. Otolaryngol. Head Neck Surg. 132(1), 37-42.

Pichichero, M.E. 2000a. Acute otitis media. Part 1. Improving diagnostic accuracy. Am. Fam. Physician 61(8), 2051-2056.

Pichichero, M.E. 2000b. Acute otitis media. Part 2. Treatment in an era of increasing antibiotic resistance. Am. Fam. Physician 61(8), 2410-2418.

Pitcher, P.A., M.M. Carr, and K.D. Clarke. 1997. Care of the child with tympanostomy tubes: a guide for the primary care physician. Dalhousie Med. J. http://medjournal.medicine.dal.ca/DMJONLIN/winter97/orig7.htm

Prescott, L.M., J.P. Harley, and D.A. Klein. 1999. Microbiology, 4th ed. McGraw-Hill, New York.

Ravics, M.E., J.J. Rosowski, and S.N. Merchant. 2004. Mechanisms of hearing loss resulting from middle ear fluid. Hear. Res. 195, 103-130.

Reinert, R.R. 2004. Pneumococcal conjugate vaccines - a European perspective. Int. J. Med. Microbiol. 294, 277-294.

Richard, C., R. Canon, K. Naghmouchi, D. Bertrand, H. Prevost, and D. Drider. 2006. Evidence on correlation between number of disulphide bridge and toxicity of class IIa bacteriocins. Food Microbiol. 23(2), 175-183.

Richelsen, B., K. Kristensen, and S.B. Pedersen. 1996. Long-term (6 months) effect of a new fermented milk product on the level of plasma lipoproteins-a placebo-controlled and double blind study. Eur. J. Clin. Nutr. 50, 811-815.

Rodriguez, J.M., M.I. Martinez, N. Horn, and H.M. Dodd. 2003. Heterologous production of bacteriocins by lactic acid bacteria. Int. J. Food Microbiol. 80, 101-106.

Roos, K., E.G. Hakansson, and S. Holm. 2001. Effect of recolonisation with "interfering" alpha streptococci on recurrences of acute and secretory otitis media in children: randomised placebo controlled trail. BMJ 322, 210-212.

Rosenfeld, R.M., and C.D. Bluestone. 1999. Evidence based otitis media. BC Becker Inc., St. Louis, MO.

Rosenfeld, R.M., and D. Kay. 2003. Natural history of untreated otitis media. In: R.M. Rosenfeld, C.D. Bluestone (Eds.). Evidence-based otitis media, 2nd ed. B.C. Becker, Hamilton, Ont. pp. 180-190.

Rosenfeld, R.M., L. Culpepper, K.J. Doyle, K.M. Grundfast, A. Hoberman, M.A. Kenna, A.S. Lieberthal, M. Mahoney, R.A. Wahl, C.R. Woods, Jr., and B.L. Yawn. 2004. Clinical practice guideline: otitis media with effusion. Otolaryngol. Head Neck Surg. 130, S95-S118.

Rossi, E.A., R.C. Vendramini, I.Z. Carlos, Y.C. Pei, and G.F. de Valdez. 1999. Development of a novel fermented soymilk product with potential probiotic properties. Eur. Food. Res. Technol. 209, 305-307.

Rovers, M.M., A.G.M. Schilder, G.A. Zielhuis, and R.M. Rosenfeld. 2004. Otitis media. Lancet 363, 465-473.

Saavedra, L., C. Minahk, A.P. De Ruiz Holgado, and F. Sesma. 2004. Enhancement of the enterocin CRL35 activity by synthetic peptide derived from NH_2-terminal sequence. Antimicrob. Agents Chemother. 48(7), 2778-2781.

Sahm, D.F., M.E. Jones, M.L. Hickey, D.R. Diakun, S.V. Mani, and C. Thornsberry. 2000. Resistance surveillance of *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* isolated in Asia and Europe 1997-1998. J. Antimicrob. Chemother. 45, 457-466.

Sanchez-Hidalgo, M., M. Maqueda, A. Galvez, H. Abriouel, E. Valdivia, and M. Martinez-Bueno. 2003. The genes coding for enterocin EJ97 production by *Enterococcus faecalis* EJ97 are located on a conjugative plasmid. Appl. Environ. Microbiol. 69, 1633-1641.

Schaberg, D.R., D.H. Culver, and R.P. Gaynes. 1991. Major trends in the microbial etiology of nosocomial infection. Am. J. Med. 91(3B), 72S-75S.

Schleifer, K.H. and R. Kilpper-Bälz. 1984. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int. J. System. Bacteriol. 34, 31-34.

Schleifer, K.H. and R. Kilpper-Bälz. 1987. Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci: a review. System. Appl. Microbiol. 10, 1-9.

Schousboe, L.P., T. Ovesen, L. Eckardt, L.M. Rasmussen, and C.B. Pedersen. 2001. How does endotoxin trigger inflammation in otitis media with effusion? Laryngoscope 111, 279-300.

Segal, N., E. Leibovitz, R. Dagan, and A. Leiberman. 2005. Acute otitis media-diagnosis and treatment in the era of antibiotic resistant bacteria: updated clinical practice guidelines. Int. J. Otorhinolaryngol. 69, 1311-1319.

Semedo, T., M.A. Santos, M.F.S. Lopes, J.J.F. Marques, M.T.B. Crespo, and R. Tenreiro. 2003. Virulence factors in food, clinical and reference enterococci: a common trait in the genus? System. Appl. Microbiol. 26, 13-22.

Seppälä, H., T. Klaukka, J. Vuopio-Varkila, A. Muotiala, H. Helenius, K. Lager, and P. Huovinen. 1997. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A Streptococci in Finland. N. Engl. J. Med. 337, 441-446.

Shankar, N., A.S. Baghdayan, and M. Gilmore. 2002. Modulation of virulence within pathogenicity island in vancomycin-resistant *Enterococcus faecalis.* Nature 417, 746-750.

Shankar, N., C.V. Lockatell, A.S. Baghdayan, C. Drachenberg, M. Gilmore, and D.E. Johnson. 2001. Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. Infect. Immun. 69, 4366-4372.

Sibold, C., J. Wang, J. Henrichsen, and R. Hakenbeck. 1992. Genetic relationship of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated from different continents. Infect. Immun. 60, 4119-4126.

Simonetta, A.C., L.G. Moragues de Velasco, and L.N. Frisón. 1997. Antibacterial activity of enterococci strains against *Vibrio cholera*. Lett. Appl. Microbiol. 24, 139-143.

Smirnova, M.G., J.P. Birchall, and J.P. Pearson. 2002. *In vitro* study of IL-8 and goblet cells: possible role of IL-8 in the aetiology of otitis media with effusion. Acta Otolaryngol. 122, 146-152.

Sokos, D.R. 2005. Pharmacists' role in increasing pneumococcal and influenza vaccination. Am. J. Health-Syst. Pharm. 62, 367-377.

Spiro, D.M., K. Tay, M.D. Baker, D.H. Arnold, and E.D. Shapiro. 2005. Wait-and-see antibiotic prescription for the treatment of acute otitis media: a randomized controlled trial. Acad. Emerg. Med. 12(5), 19.

Stein, C.R., D.J. Weber, and M. Kelley. 2003. Using hospital antibiogram data to assess regional pneumococcal resistance to antibiotics. Emerg. Infect. Dis. 2, 211-216.

Stewart, I., and P.A. Silva. 1996. Otitis media with effusion. In: Silva, P.A. and Stanton W.R. (Eds.). From Child to Adult: The Dunedin Multidisciplinary Health and Development Study. Oxford University Press, Auckland. pp. 113-129. (Reprint 1998).

Stiles, M.E., and W.H. Holzapfel. 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36, 1-29.

Sübmuth, S.D., A. Muscholl-Silberhorn, R. Wirth, M. Susa, R. Marre, and E. Rozdzinski. 2000. Aggregation substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis* within human macrophages and suppresses respiratory burst. Intect. Immun. 68, 4900-4906.

Tagg, J.R., and K.P. Dierksen. 2003. Bacterial replacement therapy: adapting 'germ warfare' to infection prevention. Trends Biotechnol. 21, 217-223.

Tanaka, A., Y. Ohashi, Y. Kakinoki, Y. Washio, K. Kishimoto, Y. Ohno, Y. Sugiura, H. Okamoto, and Y. Nakai. 1998. Influence of the allergic response on the mucociliary system in the eustachian tube. Acta Otolaryngol. Suppl. (Stockh.) 538, 98-101.

Tano, K., E. Grahn-Håkansson, S.E. Holm, and S. Hellström. 2000. Inhibition of OM pathogens by alpha-hemolytic streptococci from healthy children, children with SOM and children with rAOM. Int. J. Pediatr. Otorhinolaryngol. 56, 185-190.

Tano, K, E. Grahn-Håkansson, S.E. Holm, and S. Hellström. 2002. A nasal spray with alpha-haemolytic streptococci as long-term prophylaxis against recurrent otitis media. Int. J. Pediatr. Otorhinolaryngol. 62, 17-23.

Tano, K. C. Olofsson, E. Grahn-Håkansson, and S.E. Holm. 1999. *In vitro* inhibition of *S. pneumoniae,* non-typeable *H. influenzae* and *M. catarrhalis* by alpha-haemolytic streptococci from healthy children. Int. J. Pediatr. Otorhinolaryngol. 47, 49-56.

Thiercelin, E., and L. Jouhaud. 1903. Reproduction de l'enterocoque: Taches centrals; granulations peripheriquee et microblast. C. R. Soc. Biol. 55, 686-688. (Cited in Kalina, 1970).

Todorov, D.T., M.B. Wachsman, H. Knoetze, M. Meincken, and L.M.T. Dicks. 2005. An antibacterial and antiviral peptide produced by *Enterococcus mundtii* ST4V isolated from soya beans. Int. J. Antimicrob. Agents 25, 508-513.

Toledo-Arana, A., J. Valle, C. Solano, M.J. Arrizubieta, C. Cucarella, M. Lamata, B. Amorena, J. Leiva, J.R. Penades, and I. Lasa. 2001. The enterococcal surface protein Esp. is involved in *Enterococcus faecalis* biofilm formation. Appl. Environ. Microbiol. 67, 4538-4545.

Tomita, H., S. Fujimoto, K. Tanimoto, and Y. Ike. 1996. Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive plasmid pY117. J. Bacteriol. 178(12), 3585-3593.

Tong, M.C.F., V. Yue, P.K.M. Ku, P.S.Y. Lo, E.M.C. Wong, and C.A. van Hasselt. 2006. Risks factors for otitis media with effusion in Chinese schoolchildren: A nested case-control study and review of the literature. Int. J. Pediatr. Otorhinolaryngol. 70(2), 213-219.

Turner, D., E. Leibovitz, A. Aran, L. Piglansky, S. Raiz, A. Leiberman, and D. Alberto. 2002. Acute otitis media in infants younger than two months of age: microbiology, clinical presentation and therapeutic approach. Pediatr. Infect. Dis. J. 21(7), 669-674.

Tyrrell, G.J., R.N. Bethune, B. Willey, and D.E. Low. 1997. Species identification of enterococci via intergenic ribosomal PCR. J. Clin. Microbiol. 35, 1054-1060.

Underdahl, N.R. 1983. The effect of feeding *Streptococcus faecium* upon *Escherichia coli* induced diarrhoea in gnotobiotic pigs. Prog. Food Nutr. Sci. 7, 5-12.

Vandamme, P., B. Pot, M. Gillis, P. de Vos, K. Kersters, and J. Swings. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60, 407-438.

Vander, A., J. Sherman, and D. Luciano. 2001. Human Physiology: The mechanisms of body function, 8th ed. McGraw-Hill, New York.

Van Kempen, M.J.P., J.S. Vermeiren, M. Vaneechoutte, G. Claeys, R.H. Veenhoven, G.T. Rijkers, E.A.M. Sanders, and I.J. Dhooge. 2005. Pneumococcal conjugate vaccination in children with recurrent acute otitis media: A therapeutic alternative? Int. J. Pediatr. Otorhinolaryngol. 70(2), 275-285.

Vastag, E., H. Matthys, G. Zsamboki, D. Kohler, and G. Daikeler. 1986. Mucociliary clearance in smokers. Eur. J. Resp. Dis. 68, 107-113.

Waar, K., A.B. Muscholl-Silberhorn, R.J.L. Willems, M.J.H. Sloof, M.J.H. Harmsen, and J.E. Degener. 2002. Genotyping and incidence of virulence factors of *Enterococcus faecalis* in liver transplant patients differ from blood and faecal isolates. J. Infect. Dis. 185, 1121-1127.

Wachsman, M.B., V. Castilla, A.P. De Ruiz Holgado, R.A. de Torres, F. Sesma and C.E. Coto. 2003. Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication *in vitro*. Antiviral Res. 58, 17-24.

Wachsman, M.B., M.E. Farias, E. Takeda, F. Sesma, A.P. De Ruiz Holgado, R.A. de Torres, and C.E. Coto. 1999. Antiviral activity of enterocin CRL against herpes virus. Int. J. Antimicrob. Agents 12, 293-299.

Walls, T., D. Power, and J. Tagg. 2003. Bacteriocin-like substance (BLIS) production by the normal flora of the nasopharynx: potential to protect against otitis media? J. Med. Microbiol. 52, 829-833.

Wells, C.L., R.P. Jechorek, and S.L. Erlandsen. 1990. Evidence for the translocation of *Enterococcus faecalis* across the mouse intestinal tract. J. Infect. Dis. 162, 82-90.

Whitney, C.G., M.M. Farley, J. Hadler, L.H. Harrison, C. Lexau, A. Reingold, L. Lefkowitz, P.R. Cieslak, E.R. Zell, J.H. Jorgensen, and A. Schuchat. 2000. Increasing prevalence of multidrug-resistant *Streptococcus pneumoniae* in the United States. N. Eng. J. Med. 343, 1917-1924.

Witte, W. 1999. Antibiotic resistance in Gram-positive bacteria: epidemiological aspects. J. Antimicrob. Chemother. 44, 1-9.

Yamamoto, Y., Y. Togawa, M. Shimosaka, and M. Okazaki. 2003. Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* strain RJ-11. Appl. Environ. Microbiol. 69, 5746-5753.

Zendo, T., N. Eungruttanagorn, S. Fujioka, Y. Tashiro, K. Nomura, Y. Sera, G. Kobayashi, J. Nakayama, A. Ishizaki, and K. Sonomoto. 2005. Identification and production of a bacteriocin from *Enterococcus mundtii* QU 2 isolated from soybean. J. Appl. Microbiol. 99, 1181-1190.

Zielhuis, G.A., G.H. Rach, and P. van der Broek. 1990. The occurrence of otitis media with effusion in Dutch pre-school children. Clin. Otolaryngol. 15, 147-153.

Characterization of bacteriocin ST4SA, produced by *Enterococcus mundtii* ST4SA isolated from soya beans

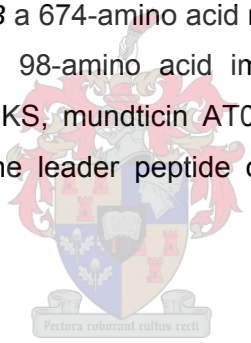
H. KNOETZE,[1] S.D. TODOROV,[1] C.A. VAN REENEN[1] AND L.M.T. DICKS[1]

[1]*Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa*

Correspondence to: Prof. L.M.T. Dicks, Department of Microbiology, University of Stellenbosch, 7600 Stellenbosch, South Africa.
Tel: +27-21-808 5849, Fax: +27-21-808 5846, e-mail: lmtd@sun.ac.za

ABSTRACT

Bacteriocin ST4SA (bacST4SA), an antimicrobial peptide produced by *Enterococcus mundtii* ST4SA isolated from soya beans, inhibits the growth of *Bacillus cereus, Clostridium tyrobutyricum, Enterococcus faecalis, Enterococcus faecium, Lactobacillus sakei, Propionibacterium* spp., *Streptococcus caprinus, Pediococcus* sp. and *Listeria monocytogenes*. No change in bacST4SA activity was recorded after 2 h of incubation at pH 2.0 to 12.0, and after 90 min at 100 °C. Fifty percent of activity was lost after 20 min at 121 °C. BacST4SA was inactivated by proteolytic enzymes and Triton X-114, but not when treated with α-amylase, Tween 20, Tween 80, Triton X-100, SDS, urea and EDTA. Maximum activity (51 200 AU/ml) was recorded in MRS broth after 14 h at 30°C. According to electron spray mass spectrometry, bacST4SA is 3.950 kDa in size. The double-glycine processing site, YGNGV consensus sequence in the N-terminal, disulphide bridge in the hydrophilic domain, and activity against *Listeria* spp. is characteristic of a class IIa bacteriocin. The genes encoding peptide ST4SA are located on a 50-kb plasmid. *MunA* encodes a 58-amino acid pre-peptide of bacST4SA, *munB* a 674-amino acid membrane-bound peptide involved in bacteriocin transport, and *munC* a 98-amino acid immunity protein. BacST4SA shares complete homology with mundticin KS, mundticin AT06, bacteriocin QU 2, and enterocin CRL35 produced by *E. mundtii*. The leader peptide of bacST4SA differs from enterocin CRL35 by two amino acids.



Bacteriocins of lactic acid bacteria (LAB) are ribosomally synthesized (1-4) and inhibit bacteria genetically closely related to the producer strain (2). These peptides are classified into four classes, with most bacteriocins grouped in classes I and II (1, 3, 5, 6). Class I peptides, known as lantibiotics (< 5 kDa), are post-translationally modified and contains lanthionine and β-methyl-lanthionine (1). Class II bacteriocins are small (< 10 kDa), heat-stable, cationic, hydrophobic and membrane-active peptides (2, 3, 7, 8). Bacteriocins with the highly conserved N-terminal amino acid sequence YGNGVXaaC (Tyr-Gly-Asn-Gly-Val- Xaa-Cys), nonpolar amino acids, one or more disulphide bridges, and activity against *Listeria* spp. are grouped in class IIa (3, 9, 10). Bacteriocins that function in pairs, usually as two distinct peptides, are grouped in class IIb (8, 10). Thiol-activated bacteriocins that rely on a *sec*-dependent secretion mechanism are grouped in class IIc (7, 10, 11).

Bacteriocins produced by *Enterococcus* spp. belong to class I, class IIa, class IIb, class IIc or class III (5, 6). Most of these bacteriocins are produced by *Enterococcus faecium* and *Enterococcus faecalis* (12). Enterocin A (13), bacteriocin N15 (14) and bacteriocin RC714 (15) produced by *E. faecium*, and enterocin SE-K4 (16) and bacteriocin 31 (17) produced by *E. faecalis* are classified as class IIa bacteriocins. Enterocins 1071A and 1071B produced by *E. faecalis* (18, 19), and enterocins L50A and L50B (20) produced by *E. faecium* are grouped in class IIb. Enterocin AS-48 (21), enterocin EJ97 (22), and enterocin RJ-11 (23) produced by *E. faecalis*, and enterocin P (24), enterocin B (25) and enterocin Q (26) produced by *E. faecium* are grouped in class IIc.

Thus far, four bacteriocins of class IIa have been described for *E. mundtii,* viz. mundticin KS (27), mundticin ATO6 (28), bacteriocin QU 2 (29) and enterocin CRL35 (30). Mundticin KS, mundticin ATO6 and bacteriocin QU 2 have identical leader and mature peptides. Although the mature peptide of enterocin CRL35 is identical to the mature peptides of the mentioned three bacteriocins, the leader peptide differs by two amino acid residues (27-30). Wachsman *et al.* (31) reported activity against herpes viruses for enterocin CRL35. This has not been reported for any of the other bacteriocins produced by *E. mundtii*.

A strain isolated from soya beans and identified as *E. mundtii* produces a bacteriocin with a broad spectrum of activity. The genes encoding bacteriocin ST4SA (bacST4SA), the immunity protein and proteins involved in secretion of the peptide (ATP-dependent transport proteins) have been sequenced.

MATERIALS AND METHODS

Isolation of lactic acid bacteria and screening for antimicrobial activity. Soya beans were homogenized in sterile physiological water, serially diluted and plated onto MRS

Agar (Biolab, Biolab Diagnostics, Midrand, SA), supplemented with 50 mg/l Natamycin (Delvocid®, Gist-brocades, B.V., Delft, The Netherlands) to inhibit fungal growth. Plates were incubated at 30 °C for 2 days and those with 50 to 100 colonies screened for antimicrobial activity according to the method described by Todorov and Dicks (32). Selected plates were overlaid with a second layer of MRS Agar supplemented with Natamycin (50 mg/l) and incubated at 30 °C for 48 h in the presence of a gas-generating kit (Oxoid, New Hampshire, England). Plates with 10 to 50 colonies were overlaid with a third layer of 10 ml semi-solid BHI medium, 1.0% (w/v) agar (Merck, Darmstadt, Germany), seeded with *Lactobacillus sakei* LMG 13558 (*ca.* 10^6 cfu/ml). Plates were incubated at 37 °C for 24 h. The test was repeated with target cells listed in Table 1. Colonies surrounded with the largest inhibition zones were selected, inoculated into MRS broth (Biolab), and re-streaked to obtain pure cultures. Antimicrobial activity was confirmed using the agar-spot method described by Ivanova *et al.* (33). Strains were stored at -80 °C in growth medium supplemented with 80% (v/v) glycerol.

Identification of strain ST4SA. Identification was by physiological and biochemical tests (34, 35, 36). Testing for pigment production and mobility were performed as described by Facklam and Collins (37) and Ball and Sellers (38), respectively. Carbohydrate fermentation reactions were recorded by using API 50 CHL and API 20 Strep test strips (Biomérieux, Marcy-l'Etiole, France). Identification was confirmed by PCR with genus- (Ent1, 5′-TACTGACAAACCATTCATGATG-3′; Ent2, 5′-AACTTCGTCACCAACGCGAAC-3′) and species-specific [1101A, 5'-AACGAGCG(A/C) (A/G)ACCC-3'; 1407R, 5'-GACGGGCGGTGTGT(A/G) C-3'] primers used in previous studies (29, 39). Cell morphology was studied by atomic force microscopy, as described by Todorov *et al.* (40).

Bacteriocin bioassay. Antimicrobial activity of strain ST4SA was confirmed by the agar-spot test and well-diffusion methods (33). To prevent the inhibitory effect of lactic acid, the pH of the supernatant was adjusted to 6.0 with sterile 1 N NaOH. Activity was expressed in arbitrary units (AU) per ml. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (33). Indicator strains are listed in Table 1.

Growth and bacteriocin production at different conditions. An 18 h-old culture of strain ST4SA was inoculated (2%, v/v, OD_{600}=2.0) into 100 ml MRS broth (Biolab), BHI broth (Merck), M17 broth (Merck), soymilk (10%, w/v, soy flour), LAPTg broth (41) supplemented with peptone from casein, 8% (w/v) molasses and 10% (w/v) molasses, respectively. Duplicate flasks were inoculated and incubated for 29 h at 30 °C and 37 °C, without agitation. Samples were taken every hour and examined for bacterial growth (OD_{600}), changes in

culture pH, and activity against *Lactobacillus casei* LHS. The agar-spot test method (33) was used and activity expressed in arbitrary units (AU) per ml, as described before.

In a separate experiment, the effect of initial medium pH on production of bacST4SA was studied. MRS broth (300 ml volumes) were adjusted to pH 4.5, 5.0, 5.5, 6.0, and 6.5, respectively, with 6 M HCl or 6 M NaOH and then autoclaved. Each flask was inoculated with 2% (v/v) of an 18 h-old culture of strain ST4SA and incubated at 30 °C for 20 h, without agitation. Changes in pH and bacST4SA production (AU/ml) were determined as described before.

Sensitivity of bacST4SA to temperature, pH, detergents, and enzymes. Strain ST4SA was grown in MRS broth (Biolab) at 30 ºC for 24 h, the cells were harvested (8000 x g, 10 min, 4 ºC) and the cell-free supernatant adjusted to pH 6.0 with 1 N NaOH. One ml samples of cell-free supernatant were exposed to 30, 60 and 100 °C, respectively, and residual bacteriocin activity tested after 10, 30 and 90 min, as described before. Activity was also tested after 20 min at 121 °C.

In a separate experiment, the effect of pH on bacST4SA activity was determined by adjusting cell-free supernatants to pH 2.0 to 12.0 (at increments of 1 pH unit) with sterile 1 N NaOH or 1 N HCl. After 2 h of incubation at room temperature, the samples were re-adjusted to pH 6.5 with sterile 1 N NaOH or 1 N HCl and tested for activity.

The effect of detergents on bacST4SA was determined by adding 1% (w/v) SDS, Tween 80, Tween 20, urea, Triton X-114 and Triton X-100, respectively, to a bacteriocin-containing cell-free supernatant. EDTA was added to a final concentration of 0.1 mM, 2.0 mM and 5.0 mM, respectively. Untreated bacteriocin-containing cell-free supernatant suspended in sterile distilled water served as control. All samples were incubated at 37 °C for 5 h and then tested for antimicrobial activity by using the agar-spot test method (33). Resistance of bacST4SA to proteolytic enzymes was determined by incubating active cell-free supernatants for 2h at 37 °C in the presence of 1.0 $mg.ml^{-1}$ and 0.1 $mg.ml^{-1}$ protease, Proteinase K, trypsin (Roche, Indianopolis, IN, USA), pronase and pepsin (Boehringer Mannheim GmbH, Germany), respectively. The effect of 0.1 and 1.0 $mg.ml^{-1}$ α-amylase (Sigma Diagnostics, St. Louis, MO, USA) on bacST4SA was tested after 2 h at 37 °C. Antimicrobial activity was determined as described before. *L. casei* LHS was used as indicator strain.

Molecular size of bacST4SA. Strain ST4SA was grown in MRS broth (Biolab) for 20 h at 30 °C. Cells were harvested (8000 x g, 10 min, 4 °C) and bacST4SA precipitated from the cell-free supernatant with 70% saturated ammonium sulphate (42). The precipitate was resuspended in one-tenth volume 25 mM ammonium acetate (pH 6.5), desalted by using a

1000 Da cutt-off dialysis membrane (Spectrum Inc., CA, USA) and separated by tricine-SDS-PAGE, as described by Schägger and Von Jagow (43). A low molecular weight marker with sizes ranging from 2.5 to 45.0 kDa (Amersham International, UK) was used. The gels were fixed and one half stained with Coomassie Blue R250 (Saarchem, Krugerdorp, South Africa). The position of the active peptide band in the gel was determined by overlaying an unstained gel with *L. casei* LHS (10^6 cfu/ml), embedded in BHI agar (Merck).

Isolation and purification of bacST4SA. Strain ST4SA was inoculated in MRS broth (Biolab), incubated for 18 h at 30 °C and then inoculated 2% (v/v) into freshly prepared MRS broth (Biolab). After 20 h of static incubation at 30 °C, the cells were harvested (8000 x g, 10 min, 4 °C) and bacST4SA precipitated from the cell-free supernatant with 80% saturated ammonium sulphate (42). The precipitate was resuspended in one-tenth-volume 25 mM ammonium acetate (pH 6.5), desalted against distilled water by using a 1000 Da cutt-off dialysis membrane (Spectrum) and loaded on a SepPak C18 column (Water Millipore, MA, USA). The column was washed with 20% (v/v) iso-propanol in 25 mM ammonium acetate (pH 6.5) and peptides eluted with 40% (v/v) iso-propanol in 25 mM ammonium acetate (pH 6.5). Fractions were collected, dried under vacuum (Speed-Vac, Savant), dissolved in 50 mM phosphate buffer (pH 6.5) and tested for antimicrobial activity as before. Active fractions were further purified by separation in a Seperdex™ 75 10/30 column (Amersham Pharmacia Biotech, Sweden) on an ÄKTA*purifier* (Amersham). Peptides were eluted from the column with 50 mM phosphate buffer and 400 mM NaCl (pH 6.5), and detected at 254 and 280 nm. Fractions containing active peptide were collected, dried under vacuum and stored at -20 °C. Activity was tested by using the agar-spot test method, as described before.

Mass spectrometry. Active fractions collected from the ÄKTA*purifier* were subjected to electro-spray mass spectrometry (ES-MS). A VG Bio-Q quadrupole with a mass range of 4000 Da (Bio-Tech, Manchester, UK) in the positive mode was used. The protein was dissolved in H_2O/CH_3CN (50/50, v/v) and 1.0% acetic acid at a concentration of approximately 5 pmol/µl (by volume). Ten µl-aliquots were introduced into the ion source at a flow rate of 4 µl/min. Scanning was performed from m/z = 500 to 1.500 in 10 s, with the resolution adjusted so that the peak at m/z = 998 from horse heart myoglobin was 1.5–2 wide on the base. Calibration was performed by using the multiply charged ions produced by separate injections with horse heart myoglobin (16950.4 Da), as described by Jaquinod *et al.* (44).

Analysis of the bacST4SA operon. Total genomic DNA was extracted according to the method described by Dellaglio *et al.* (45). Plasmid DNA was isolated according to Burger and Dicks (46), followed by CsCl density gradient centrifugation (47). DNA was separated on a 0.6% (w/v) agarose gel, according to Ausubel *et al.* (47). Genomic and plasmid DNA were used as templates. The structural gene encoding bacST4SA was amplified using DNA primers designed from the sequence of bacteriocin QU 2 (29). Two sets of primers were designed for amplification of the ATP-dependent transporter gene and one set for the amplification of the immunity gene, all based on sequences of the mundticin KS operon (GenBank Accession number AB066267). Primers and their base pair sequences are listed in Table 2.

Amplification of the ATP-dependent transporter and immunity genes was performed with *Taq* DNA polymerase in the following settings: denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and polymerization at 72 ° for 7 min. Amplified fragments were separated on a 1% (w/v) agarose gel as before. A 50-bp DNA fragment (Amersham Bioscience, UK Limited, UK) was used as marker. Amplified products were purified using the QIAquick® PCR purification kit (Qiagen, Valencia, California, USA). Purified fragments were cloned into pGEM®-T Easy Vector (pGEM®-T Easy Systems, Promega, Madison, USA). Plasmid isolations were performed on selected transformants, using the QIAquick® PCR purification kit (Qiagen). Fragments were purified as described before and sequenced using the BigDye™ Terminator Cycle (Biosystems, Warrington, England) on an ABI Genetic Analyser 3130XL sequencer (Applied Biosystem, SA Pty Ltd.).

Correlation of DNA and amino acid sequences. The BLAST program of the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/), and DNAMAN software for Windows® (Lynnon Biosoft, Vaureuil, Quebec, Canada) were used to obtain amino acid-, DNA sequences, and hydrophobicity profiles. The membrane spanning of the ATP-dependent transporter peptide of bacST4SA was predicted using the SOSUI transmembrane prediction program (48).

Location of the bacST4SA gene cluster. Plasmid DNA was isolated from strain ST4SA as described before. A transposon with an R6K*y* origin of replication and a kanamycin resistance gene was inserted into the plasmid DNA, using the EZ::TN™ <R6K*y*ori / KAN-2> Insertion Kit (Epicenter®, Madison, Wisconsin). The transposed plasmid DNA was transformed to *Escherichia coli* EC100D (*rec*A$^-$, *pit*+) and plated onto LB Agar (Merck), supplemented with kanamycin (50 µg/ml). After 12 h of incubation at 37 °C, 24 colonies were randomly selected and plasmid DNA isolated as described before. Plasmids were digested

with *Eco*RI and *Eco*RV (Roche). Plasmid DNA with the transposon was amplified using the primers listed in Table 2 and primers described by Zendo *et al.* (29). The amplified DNA products were separated as described previously.

RESULTS

Identification of strain ST4SA. Strain ST4SA is a Gram-positive, non-motile, catalase-negative coccus (Fig. 1). Growth was recorded at 10 and 45 °C, pH 4.4 and 9.6, and in the presence of 6.5% (w/v) NaCl. L-lactic acid is produced from D-glucose. Sugar fermentation reactions corresponded to that described for the genus *Enterococcus* (49). Identification at genus-level was confirmed by amplification of a 112-bp PCR-fragment (Fig. 2). Identification as *E. mundtii* was confirmed by a 306-bp fragment amplified with species-specific primers (Fig. 3).

Spectrum of activity. BacST4SA inhibited the growth of *B. cereus, C. tyrobutyricum, E. faecalis, E. faecium, L. sakei, Pediococcus* sp., *Propionibacterium* spp., *S. caprinus* and *L. monocytogenes* (Table 1). No activity was observed against *Clostridium sporogenes*, other *Lactobacillus* spp., *Leuconostoc mesenteriodes* subsp. *cremoris*, *Pediococcus pentosaceus*, *Propionibacterium acidipropioni* and *Streptococcus thermophilus* (Table 1).

Growth and bacST4SA production at different conditions. Maximum activity of bacST4SA (51 200 AU/ml) was recorded after 14 h of growth in MRS broth at 30 °C (Fig. 4). During 29 h of growth, the culture pH decreased to 4.60 and the optical density (OD_{600}) increased from 0.03 to approximately 2.5 (Fig. 4). Low levels (1 600 AU/ml) of bacST4SA were recorded in M17 broth and soymilk. BacST4SA activity of 3 600 AU/ml was recorded in molasses and 12 800 AU/ml in LAPTg broth supplemented with peptone from casein (not shown).

No change in culture pH was recorded when strains were grown in MRS adjusted to pH 4.5. However, the culture pH decreased to 4.8 when cells were grown in MRS with an initial pH of 5.0 and 6.0. BacST4SA levels of 3 200 AU/ml were recorded in MRS broth with an initial pH of 4.5 and 5.0. Production increased to 6 400, 25 600 and 51 200 AU/ml in MRS broth with an initial pH of 5.5, 6.0 and 6.5, respectively (not shown).

Effect of temperature, pH, detergents and enzymes on bacteriocin activity. Complete inactivation of antibacterial activity was observed after treatment of bacST4SA-containing cell-free supernatant with proteolytic enzymes (not shown). No change in activity

was recorded when treated with α-amylase, after 2 h of incubation at pH 2.0 to 12.0, and after 90 min of treatment at 30, 60 and 100 °C, respectively. However, bacST4SA activity decreased by approximately 50% after 20 min at 121 °C. BacST4SA remained stable when treated with Triton X-100, SDS, Tween 20, Tween 80, urea (1%, v/v, final concentration), and EDTA (1.0, 2.0 and 5.0 mM). No activity was recorded after treatment with 1% (v/v) Triton X-114.

Purification and molecular size of bacST4SA. Separation by tricine-SDS-PAGE yielded an active peptide band between 2.35 and 3.4 kDa (Fig. 5). Eighty percent of bacST4SA was recovered with ammonium sulphate precipitation. Active fractions collected from the ÄKTA*purifier* corresponded to a peak that eluted at 43 - 47 min (Fig. 6A). Re-injection of the pooled fractions yielded an active peptide peak that eluted at 45 - 50 min (Fig. 6B). Separation by ES-MS yielded a distinctive peak corresponding to 3 950 Da (Fig. 7).

Analysis of the bacST4SA operon. Primers designed from the structural gene of bacteriocin QU 2 yielded a 380 bp fragment, designated as *munA* (Fig. 8A). DNA fragments of 914 and 974 bp were amplified with primers designed from the ATP-dependent transporter genes described for mundticin KS and were named *munB* (Fig. 8B). An immunity gene of 767 bp (*munC*) was amplified with primers designed from the immunity gene of mundticin KS (Fig. 8B).

Correlation of the sequences on the BLAST protein database revealed that *munA* encodes a 58-amino acid prepeptide, which contains a 15-amino acid leader peptide with a double-glycine processing site (Fig. 9A). The YGNGV consensus motif and a disulphide bridge linking cys^{9} and cys^{14} were detected (Fig. 9A). Alignment of the amino acid sequences revealed complete homology between the leader and mature peptides of bacST4SA and mundticin KS (27), mundticin AT06 (28) and bacteriocin QU 2 (29). BacST4SA is completely homologous to the mature peptide enterocin CRL35 (30). However, the leader peptide of bacST4SA differs from the leader peptide of enterocin CRL35 by two amino acids, at positions -6 (A to S) and -10 (S to A). The leader and mature peptides of bacST4SA are amphiphilic, consisting of hydrophobic and polar, hydrophilic residues (Fig. 9A).

The immunity gene encoding the 98-amino acid protein (Fig. 9B) is completely homologous to the immunity protein of enterocin CRL35 and 96.9% homologous to the immunity protein of mundticin KS, with differences at positions 30, 47, and 89 (Fig. 9B).

The 674-amino acid ATP-dependent transporter protein of bacST4SA is 98.9% homologous to mundticin KS and 99.3% to enterocin CRL35 (Fig. 10). Differences occurred at amino acid positions 107, 353, 494, 616, 642, 654, and 665 for mundticin KS, and at positions 108, 386, 579, 585, and 639 for enterocin CRL35 (Fig. 10). The ATP-dependent

transporter gene consists of three domains responsible for bacST4SA translocation (Fig. 10). The peptidase C39B domain (amino acid 1 to 25) contains a bacteriocin-processing site (Fig. 10). Amino acid 137 - 415 represents the ABC-membrane transporter domain with several transmembrane helixes (Fig. 10). Amino acids 461 - 674 are part of the ABC-DLP family (Fig. 10). Analysis of the ABC-transporter domain, using the SOSUI database, revealed five primary transmembrane helixes and one secondary transmembrane helix (Table 3). The membrane helixes have a number of hydrophobic residues and polar residues, and a few uncharged and charged residues (Table 3).

The N-terminal region of the bacST4SA mature peptide is hydrophilic to amphiphilic, while the central and C-terminal regions are hydrophobic to amphiphilic (Fig. 11).

Location of bacST4SA gene cluster. Two plasmids of approximately 50 and 100 kb were isolated from strain ST4SA (Fig. 12A). From the 24 clones selected for plasmid isolation, one contained the recombinant plasmid. Amplification of the 50-kb plasmid with primers designed to detect the structural-, ATP-dependent transporter- and immunity genes of bacST4SA yielded 380 bp, 914 and 974 bp, and 767 bp fragments, respectively (Fig. 12B). The restriction enzyme banding patterns of plasmid containing the EZ::TN <R6Kγori/KAN-2> transposon and native plasmid were similar (Fig. 12C).

DISCUSSION

Strain ST4SA is identified as *E. mundtii*, based on phenotypic and genetic characteristics. The morphology is typical of that described for the genus *Enterococcus*. The strain is non-motile, which distinguishes it from the yellow-pigmented species *Enterococcus casseliflavus* (49).

BacST4SA inhibited the growth of *B. cereus, C. tyrobutyricum, E. faecalis, E. faecium, L. sakei, Pediococcus* sp., *Propionibacterium* spp., *S. caprinus* and *L. monocytogenes.* The spectrum of activity of bacST4SA differs from that reported for enterocin A, mundticin KS and bacteriocin QU 2 by being active against *Bacillus cereus,* but not *Lactobacillus plantarum* (13, 27, 29). Concluded from the spectrum of activity, bacST4SA is closer related to enterocin P. However, enterocin P inhibits *Lactobacillus fermentum* and *Lactobacillus curvatus* (24), whilst bacST4SA does not. BacST4SA and mundticin KS inhibits *E. faecium*, whilst bacteriocin QU 2 does not (27, 29).

The stability of bacST4SA at acidic and alkaline pH (pH 2 to 12) and at high temperatures (completely stable for 90 min at 100 °C and 50% reduction of activity after 15 min at 121 °C) distinguishes the peptide from other enterocins in class IIa. Enterocin P (24),

enterocin RC714 (15) and bacteriocin N15 (14) lost activity after autoclaving (15 min, 121 °C). Enterocin SE-K4 and mundticin KS remained active after incubation at pH 3 - 11, but lost 50% activity after 60 min at 100 °C (16, 27). Mundticin AT06 and enterocin CRL35 lost activity after 15 min at 100 °C (28, 30). Enterocin CRL35 is unstable at pH 3 and below (30). Bacteriocin QU 2 remains active between pH 2 and 11, but looses most of its activity at pH 12. Bacteriocin QU 2 is heat resistant, depending on pH (29). The peptide remained active after 10 min at 100 °C when suspended at pH 3 and 5, but lost most of its activity under these conditions at pH 7 and 10 (29).

Treatment of bacST4SA against a variety of proteolytic enzymes resulted in a complete loss in activity, confirming its proteinaceous nature. Similar results were reported for mundticin AT06 (28), mundticin KS (27), bacteriocin QU 2 (29), bacteriocin N15 (14), enterocin P (24) and bacteriocin RC714 (15). Resistance to α-amylase suggests that bacST4SA is not glycosylated and that it does not depend on a carbohydrate moiety for activity.

No changes in bacST4SA activity were recorded when treated with Triton X-100, SDS, Tween 80, Tween 20 and EDTA. However, treatment with Triton X-114 resulted in a complete loss of activity. Triton X-114 is used to isolate soluble proteins from lipids and carbohydrates (47). Loss in activity suggests that bacST4SA has been dissolved in the Triton X-114 fraction. The effect of urea and EDTA on mundticin AT06 (28), mundticin KS (27), bacteriocin QU 2 (29) and enterocin CRL35 (30) is unknown. It is, however, unlikely that mundticin KS, mundticin AT06 and bacteriocin QU 2 are affected by SDS, since active peptide bands were obtained after tricine-SDS gel electrophoresis (27-29). Tween 20 and Tween 80 had no effect on bacST4SA production. However, Zendo *et al.* (29) reported an increase in activity of bacteriocin QU 2 when treated with Tween 80. This surfactant increases the activity of bacteriocins by reducing adsorption to the producer strain, glass surfaces and polypropylene (50-52).

Low levels of bacST4SA activity were recorded when the strain was cultured in M17 broth, BHI broth, molasses and soymilk, despite relative good cell growth. This suggests that specific nutrients are required for optimal bacteriocin production. Maximum production of bacST4SA (51 200 AU/ml) was recorded after 14 h of growth in MRS broth (Fig. 4). Low levels of bacST4SA were detected after 4 h of growth, suggesting that the peptide is a primary metabolite. Similar results have been reported for other class IIa enterocins. Maximum activity was recorded for enterocin P and bacteriocin N15 during late exponential phase, followed by a decrease during stationary growth (24, 14). Maximum activity of bacteriocin SE-K4 was recorded at 43 and 45 °C (16), while maximum activity of mundticin KS was recorded during early stationary growth at 15 to 40 °C, but not at 45 °C (27).

The culture pH of strain ST4SA decreased from 6.0 to 4.9 during growth in MRS broth. BacST4SA production in the same medium, adjusted to pH 4.5 and 5.0 was relatively slow. However, optimal production was recorded at pH 5.5 and higher. This suggests that bacST4SA accumulates during the first phase of growth before the pH decreases to 5.0. Production of bacteriocin QU 2 continued during stationary growth when cells were inoculated in medium with a pH of 5.0 or 6.0 (29). Maximum activity (204 800 AU/ml) was recorded in medium adjusted to pH 6.0 (29). Production of bacteriocin QU 2 declined drastically during stationary growth when cells were inoculated in medium of pH 7.0 (29).

BacST4SA has an estimated molecular mass of 3.950 kDa and is smaller than mundticin KS (4.290 kDa), mundticin AT06 (4.287 kDa) and bacteriocin QU 2 (4.287 kDa), but larger than enterocin CRL35 (3.500 kDa) (27-30). The differences recorded in molecular sizes might be due to buffers used during purification and mass spray spectrometry. BacST4SA has a theoretical molecular mass of 4271.73 Da.

The bacST4SA operon consists of three genes, *viz. munA* encoding a soluble 58-amino acid pre-peptide (Fig. 9A), *munB* encoding a 674-amino acid ATP-dependent transporter peptide (Fig. 10) located in the cell membrane, and *munC* encoding a soluble 98-amino acid immunity peptide (Fig. 9B).

The YGNGV consensus motif is considered to be part of a recognition sequence for a speculated membrane-bound protein receptor (53). BacST4SA is translated as a pre-peptide consisting of double-glycine amino acids at position -1 and -2, unique to class IIa bacteriocins (10). The double-glycine leaders generally serve as a signal peptide for a *sec*-independent ABC-transporter (10). BacST4SA also contains one disulphide bridge, linking cys^{9} and cys^{14}, not post-translationally modified. In class IIa bacteriocins, cysteine amino acid molecules form cross-linked, covalently disulphide bridges to maintain intramolecular rigidity (10, 54). The high number of non-polar amino acids renders a high degree of conformational freedom to bacST4SA (55). Polar, hydrophilic amino acid residues form binding sites with charged molecules (54). The bacST4SA ATP-dependent transporter peptide consists of 6 bundles of hydrophobic transmembrane helices (Table 3). These helices are stabilized by a combination of amphiphilic side chains and polar side chain interactions (48). Similar conformations were recorded for enterocin P, consisting of hydrophobic transmembrane segments supported by highly hydrophobic transmembrane segments at the C-terminal, possibly responsible for insertion of the peptide into the cytoplasm membrane (24). The ATP-dependent transporter also contained a peptidase C39B domain, responsible for cleavage of the double-glycine leader peptide from the precursors of class IIa bacteriocins, mediating secretion of the peptide and transport across the cytoplasmic membrane (56). The ATP-DPL domain is involved in lipid, peptide and drug export (57).

The mature bacteriocin (bacST4SA) is completely homologous to mundticin KS (27), mundticin AT06 (28), bacteriocin QU 2 (29) and enterocin CRL35 (30). The genes encoding these bacteriocins are located on plasmids and horizontal gene transfer may have occurred. The broad spectrum of activity recorded for bacST4SA, compared to mundticin KS, mundticin AT06, bacteriocin QU 2 and enterocin CRL35 remains unknown.

ACKNOWLEDGEMENT

This research was funded by Cipla Medpro (Pty) Ltd, Shephard Medical (Pty) Ltd and the National Research Foundation (NRF), South Africa. Mass spectrometry was performed by Dr. A. Venter, Central Analytical Facility, University of Stellenbosch. M. Matthews provided technical assistance with the ÄKTA*purifier*. We would like to thank Dr. S. M. Deane and Mr. L.J. van Zyl, Department of Microbiology, University of Stellenbosch, for critical discussions and their assistance in the isolation of plasmid DNA.

REFERENCES

1. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:**39-86.

2. **De Vuyste, L., and E. J. Vandamme.** 1994. Antimicrobial potential of lactic acid bacteria, p. 91-142. *In* L. De Vuyst, and E. J. Vandamme (ed.), Bacteriocins of lactic acid bacteria: Microbiology, Genetics and Application, Blackie Academic and Professional, London, UK.

3. **Nes, I. F., B. D. Diep, L. S. Havarstein, M. B. Brurberg, V. Eijsink, and H. Holo.** 1996. Biosynthesis of bacteriocins of lactic acid bacteria. Antonie van Leeuwenhoek **70:**113-128.

4. **Nissen-Meyer, J., and I. F. Nes.** 1997. Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. Archives Microbiol. **167:**67-77.

5. **Devriese, L. A., and B. Pot.** 1995. The genus *Enterococcus*, p. 327-367. *In* B. J. B. Wood, and W. H. Holzapfel (ed.), The genera of lactic acid bacteria, Blackie Academic and Professional, London.

6. **Franz, C. M. A. P., and W. H. Holzapfel.** 2004. The genus *Enterococcus*: biotechnological and safety issues, p. 199-247. *In* S. Salminen, A. von Wright, and A. Ouwenhand (ed.), Lactic acid bacteria: microbiological and functional aspects, 3rd ed. Marcel Dekker Inc., New York.

7. **Abee, T., L. Krockel, and C. Hill.** 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. Int. J. Food Microbiol. **28:**169-185.

8. **Rodriguez, J. M., M. I. Martinez, N. Horn, and H. M. Dodd.** 2003. Heterologous production of bacteriocins by lactic acid bacteria. Int. J. Food Microbiol. **80:**101-106.

9. **Eijsink, V. G., M. Skeie, P. H. Middelhoven, M. B. Brurberg, and I. F. Nes.** 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. **64:**3275-3281.

10. **Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki.** 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. **24:**85-106.

11. **Héchard, Y., and H-G. Sahl.** 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie **84:**545-557.

12. **Moreno, M. R. F, P. Sarantinopoulos, E. Tsakalidou, and L. de Vuyst.** 2005. The role and application of enterococci in food and health. Int. J. Food Microbiol. **106(1):**1-24.

13. **Aymerich, T., H. Holo, L. S. Havarstein, M. Hugas, M. Garriga, and I. F. Nes.** 1996. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl. Environ. Microbiol. **62:**1676-1682.

14. **Losteinkit, C., K. Uchiyama, S. Ochi, T. Takaoka, K. Nagahisa, and S. Shioya.** 2001. Characterization of bacteriocin N15 produced by *Enterococcus faecium* N15 and cloning of the related genes. Biosci. Bioeng. **91(4):**390-395.

15. **del Campo, R., C. Tenorio, R. Jiménez-Diaz, C. Rubio, R. Gomez-Lus, F. Baquero, and C. Torres.** 2001. Bacteriocin production in vancomycin-susceptible *Enterococcus* isolates of different origin. Antimicrob. Agents Chemoch. **45:**905-912.

16. **Eguchi, T., K. Kaminaka, J. Shima, S. Kawamoto, K. Mori, S-H. Choi, K. Doi, S. Ohmomo, and S. Ogata.** 2001. Isolation and characterization of enterocin SE-K4 produced by thermophilic enterococci, *Enterococcus faecalis* K4. Biosci. Biotechnol. Biochem. **65:**247-253.

17. **Tomita, H., S. Fujimoto, K. Tanimoto, and Y. Ike.** 1996. Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive plasmid pY117. J. Bacteriol. **178(12):**3585-3593.

18. **Balla, E., L. M. T. Dicks, M. du Toit, M. J. van der Merwe, and W. H. Holzapfel.** 2000. Characterization and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by *Enterococcus faecalis* BFE 1071. Appl. Environ. Microbiol. **66:**1298-1304.

19. **Balla, E., and L. M. T. Dicks.** 2005. Molecular analysis of the gene cluster involved in the production and secretion of enterocins 1071A and 1071B and of the genes responsible for the replication and transfer of plasmid pEF1071. Int. J. Food Microbiol. **99(1):**33-45.

20. **Cintas, L. M., P. Cassaus, H. Holo, P. E. Hernandez, I. F. Nes, and L. S. Havarstein.** 1998. Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to staphylococcal hemolysins. J. Bacteriol. **180:**1988-1994.

21. **Martinez-Bueno, M. M., M. Maqueda, A. Galvez, B. Samyn, J. van Beeumen, J. Coyette, and E. Valdivia.** 1994. Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48. J. Bacteriol. **176:**6334-6339.

22. **Galvez, A., E. Valdivia, H. Abriouel, E. Camafeita, E. Mendez, M. Martinez-Bueno, and M. Maqueda.** 1998. Isolation and characterization of enterocin EJ97, a bacteriocin produced by *Enterococcus faecalis* EJ97. Archives Microbiol. **171:**59-65.

23. **Yamamoto, Y., Y. Togawa, M. Shimosaka, and M. Okazaki.** 2003. Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* strain RJ-11. Appl. Environ. Microbiol. **69:**5746-5753.

24. **Cintas, L. M., P. Casaus, L. S. Havarstein, P. E. Hernández, and I. F. Nes.** 1997. Biochemical and genetic characterization of enterocin P, a novel *sec*-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. **63:**4321-4330.

25. **Casaus, P., T. Nilsen, L. M. Cintas, I. F. Nes, P. E. Hernández, and H. Holo.** 1997. Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. Microbiol. **143:**2287-2294.

26. **Cintas, L. M., P. Casaus, C. Herranz, L. S. Havarstein, H. Holo, P. Hernandez, and I. F. Nes.** 2000. Biochemical and genetic evidence that *Enterococcus faecium* L50 produces enterocins L50A and L50B, *sec*-dependent enterocin P, and a novel bacteriocin secreted without a N-terminal extension termed enterocin Q. J. Bacteriol. **182:**6806-6814.

27. **Kawamoto, S., J. Shima, R. Sato, T. Educhi, S. Ohmomo, J. Shibato, N. Horikashi, K. Takeshita, and T. Sameshima.** 2002. Biochemical and genetic characterization of mundticin KS, an antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. Appl. Environ. Microbiol. **68:**3830-3840.

28. **Bennik, M. H. J., B. Vanloo, R. Brasseur, L. G. M. Gorris, and E. J. Smid.** 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: Full characterization and interaction with target organism. Biochem. Biophys. Acta **1373:**47-58.

29. **Zendo, T., N. Eungruttanagorn, S. Fujioka, Y. Tashiro, K. Nomura, Y. Sera, G. Kobayashi, J. Nakayama, A. Ishizaki, and K. Sonomoto.** 2005. Identification and production of a bacteriocin from *Enterococcus mundtii* QU2 isolated from soybean. J. Appl. Microbiol. **99:**1181-1190.

30. **Saavedra, L., C. Minahk, A. P. de Ruiz Holgado, and F. Sesma.** 2004. Enhancement of the enterocin CRL35 activity by synthetic peptide derived from the NH_2-terminal sequence. Antimicrob. Agents Chemother. **48:**2778-2781.

31. **Wachsman, M. B., M. E. Farias, E. Takeda, F. Sesma, A. P. De Ruiz Holgado, R. A. de Torres, and C. E. Coto.** 1999. Antiviral activity of enterocin CRL35 against herpesvirus. Int. J. Antimicrob. Agents **12:**293-299.

32. **Todorov, S. D., and L. M. T. Dicks.** 2004. Screening of lactic-acid bacteria from South African barley beer for production of bacteriocin-like compounds. Folia Microbiol. **49:**406-410.

33. **Ivanova, I., V. Miteva, Ts. Stefanova, A. Pantev, I. Budakov, S. Danova, P. Moncheva, I. Nikolova, X. Dousset, and P. Boyaval.** 1998. Characterization of a bacteriocin produced by *Streptococcus thermophilus* 81. Int. J. Food. Microbiol. **42:**147-158.

34. **Schillinger, U., and F. K. Lücke.** 1987. Identification of lactobacilli from meat and meat products. Food Microbiol. **4:**199-208.

35. **Stiles, M. E., and W. H. Holzapfel.** 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food. Microbiol. **36:**1-29.

36. **Collins, M. D., B. A. Phillips, and P. Zanoni.** 1989. Deoxyribonucleic acid homology studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. *nov*., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. *nov*., comb. nov. Int. J. Syst. Bacteriol. **39:**105-108.

37. **Facklam, R. R., and M. D. Collins.** 1989. Identification of *Enterococcus* species isolated from human infections by a conventional tests scheme. J. Clin. Microbiol. **27:**731-734.

38. **Ball, R. J., and W. Sellers.** 1966. Improved motility medium. Appl. Microbiol. **14:**670-673.

39. **Ke, D., F. J. Picard, F. Martineau, C. Ménard, P. H. Roy, M. Onellette, and M. G. Bergeron.** 1999. Development of PCR assay for rapid detection of enterococci. J. Clin. Microbiol. **37:**3497-3503.

40. **Todorov, D. T., M. B. Wachsman, H. Knoetze, M. Meincken, and L. M. T. Dicks.** 2005. An antibacterial and antiviral peptide produced by *Enterococcus mundtii* ST4V isolated from soya beans. Int. J. Antimicrob. Agents **25:**508-513.

41. **Raibaud, P., M. Caulet, J. V. Galpin, and G. Mocquot.** 1961. Studies on the bacterial flora of the alimentary tract of pigs. II. Streptococci: selective enumeration and differentiation of the dominant group. J. Appl. Bacteriol. **24:**285-305.

42. **Sambrook, J. E., F. Eritsch, and J. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold spring Harbour, Cold spring harbour Laboratory Press, N.Y.

43. **Schägger, H., and G. Von Jagow.** 1987. Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of protein in the range from 1 to 100 kDa. Anal. Biochem. **166:**368-379.

44. **Jaquinod, M., N. Pottier, K. Klarskov, J. M. Reymann, O. Sorokine, S. Kieffer, P. Barth, V. Andriantomanga, J. F. Bielmann, and V. Dorsselaer.** 1993. Sequence of pig lens aldose reductase and electrospray mass spectrometry of non-covalent and covalent complex. Eur. J. Biochem. **218:**893-903.

45. **Dellaglio, F. V., Bottazzi, and L. D. Trostelli.** 1973. Deoxyribonucleic acid homology and vase composition in some thermophilic lactobacilli. J. Gen. Microbiol. **74:**289-297.

46. **Burger, J. H., and L. M. T. Dicks.** 1994. Technique for isolating plasmids from exopolysaccharide producing *Lactobacillus* spp. Biotechnol. Tech. **8:**769-772.

47. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1994. Current Protocols in Molecular Biology, John Wiley and Sons, Inc., New York.

48. **Hirokawa, T., S. Boon-Chieng, and S. Mitaku.** 1998. SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics **14:**378-379.

49. **Holt, J.G.** 1994. Bergey's manual of determinative bacteriology. Williams and Wilkens, Baltimore.

50. **Aymerich, T., M. G. Artigas, M. Garriga, J. M. Monfort and M. Hugas.** 2000. Effect of sausage ingredience and additives on the production of enterocin A and B by *Enterococcus faecium* CTC492. Optimization of in vitro production and anti-listerial effect in dry fermented sausages. J. Appl. Microbiol. **88:**686-694.

51. **Keren, T., M. Yarmus, G. Halevy, and R. Shapira.** 2004. Immunodetection of the bacteriocin lactivin RM: analysis of the influence of temperature and Tween 80 on its expression and activity. Appl. Environ. Microbiol. **70:**2098-2104.

52. **Joosten, H. M. L. J., and M. Nuñez.** 1995. Adsorption of nisin and enterocin 4 to polypropylene and glass surfaces and its prevention by Tween 80. Lett. Appl. Microbiol. **21:**389-392.

53. **Fleury, Y., M. Abdel Dayem, J. J. Montagne, E. Chaboisseau, J. P. Le Caer, P. Nicolas, and A. Delfour.** 1996. Covalent structure, synthesis, and structure-function studies of mesentericin Y 10537, a defensive peptide from Gram-positive bacteria *Leuconostoc mesenteroides.* J. Biol. Chem. **271:**14421-14429.

54. **Lodish, H., A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore, and J. Darnell.** 1999. Molecular cell biology, 4th ed. W. H. Freeman and Company, New York.

55. **Kaiser, A. L., and T. J. Montville.** 1996. Purification of the bacteriocin bavaricin MN and characterization of its mode of action against *Listeria monocytogenes* Scott A cells and lipid vesicles. Appl. Environ. Microbiol. **62:**4529-4535.

56. **Dirix, G., P. Monsieurs, B. Dombrecht, R. Daniels, K. Marchal, J. Vanderleyden, and J. Michiels.** 2004. Peptide signal molecules and bacteriocins in Gram-negative bacteria: a genome-wide in silico screening for peptides containing a double-glycine leader sequence and their cognate transporters. Peptides **25:**1425-1440.

57. **Marchler-Bauer, A., and S. H. Bryant.** 2004. CD-Search: protein domein annotations on the fly. Nucleic Acids Res. **32(W):**327-331.

58. **Cintas, L. M., J. M. Rodriguez, M. F. Fernandez, K. Sletten, I. F. Nes, P. E. Hernandez, and H. Holo.** 1995. Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad spectrum. Appl. Environ. Microbiol. **61:**2643-2648.

TABLE 1. Antimicrobial activity of bacST4SA in pH-neutralized cell-free supernatant

Target strain	Temperature (°C)	Growth medium, incubation	Antimicrobial activity[a]
Bacillus cereus LMG 13569	37	BHI, aerobic	++
Clostridium sporogenes LMG 13570	37	RCM, anaerobic	-
Clostridium tyrobutyricum LMG 13571	30	RCM, anaerobic	++
Enterococcus faecalis 20, 21, E77, E80, E90, E92, FA2, LMG 13566	37	MRS, aerobic	++
Enterococcus faecium HKLHS	30	MRS, aerobic	++
Lactobacillus acidophilus LMG 13550	37	MRS, anearobic	-
Lactobacillus bulgaricus LMG 13551	37	MRS, anaerobic	-
Lactobacillus casei LMG 13552	37	MRS, anaerobic	-
Lactobacillus carvatus LMG 13553	30	MRS, anaerobic	-
Lactobacillus fermentum LMG 13554	37	MRS, anaerobic	-
Lactobacillus helveticus LMG 13555	42	MRS, anaerobic	-
Lactobacillus plantarum LMG 13556	37	MRS, anaerobic	-
Lactobacillus reuteri LMG 13557	37	MRS, anaerobic	-
Lactobacillus sakei LMG 13558	30	MRS, anaerobic	++
Leuconostoc mesenteroides subsp. *cremoris* LMG 13562	30	MRS, anaerobic	-
Listeria innocua LMG 13568	30	BHI, aerobic	-
Listeria monocytogenes	30	BHI, aerobic	++
Pediococcus sp.	30	MRS, anaerobic	++
Pediococcus pentosaceus LMG 13560	30	MRS, anearobic	-
Propionibacterium acidipropionici LMG 13572	32	GYE, anaerobic	-
Propionibacterium sp. LMG 13574	32	GYE, anaerobic	++
Streptococcus caprinus TS1, TS2	37	BHI, aerobic	++
Streptococcus thermophilus LMG 13565	42	MRS, anaerobic	-

[a] = no activity; + = activity zones of at least 5 mm in diameter; ++ = activity zones of at least 6 mm in diameter. LMG = Laboratorium voor Microbiologie, University of Ghent, Belgium. The other strains were from own culture collection. GYE = glucose yeast extract, prepared according to Cintas *et al.* (58). The other growth media were from Biolab, Biolab Diagnostics, Midrand, South Africa.

TABLE 2. DNA primers used in this study

Gene	DNA primer	Description	Fragment size (bp)
ABC-transporter$_{N\text{-terminal}}$	ABC1-F ABC1-R	TGATGGATTTCAGTGGAAGT ATCTCTTCTCCGTTTAATCG	914
ABC-transporter$_{C\text{-terminal}}$	ABC2-F ABC2-R	GTCATTGTTGTGGGGATTAT TCTAGATACGTATCAAGTCC	974
Immunity gene	munC-F munC-R	TTCCTGATGAACAAGAACTC GTCCCCACAACCAATAACTA	767

TABLE 3. Amino acid sequence of a membrane ABC transporter protein consisting of six transmembrane helices

N-terminal position	Transmembrane region	C-terminal	Type	Length
147	LVIL**SS**LII**T**IIGL**SSYY**F*R*ILI	169	Primary	23
179	FL**N**LFMI**S**I**SY**IIGIFI**TS**IFEI	201	Primary	23
214	VG*R***S**ILF*K***Y**LE*H*IFIL**P**A**S**FF**S**	235	Secondary	22
254	EALA**S**F**T**I**S**IFLDL**SS**VIVVGII	276	Primary	23
282	*K***Q**LFLI**T**L**SS**I**P**F**Y**ILIILG**SN**	303	Primary	22
370	**N**L*K*ILV**S**LL**TS**AFVL**W**FG**SYY**VI	392	Primary	23

Bold = polar residues; non-bold = hydrophobic residues; underline = negatively charged residues; italics = positively charged residues

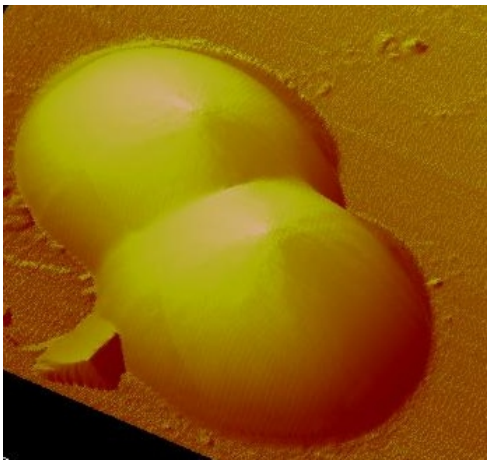


FIG. 1. Morphology of strain ST4SA, visualized by atomic force microscopy.

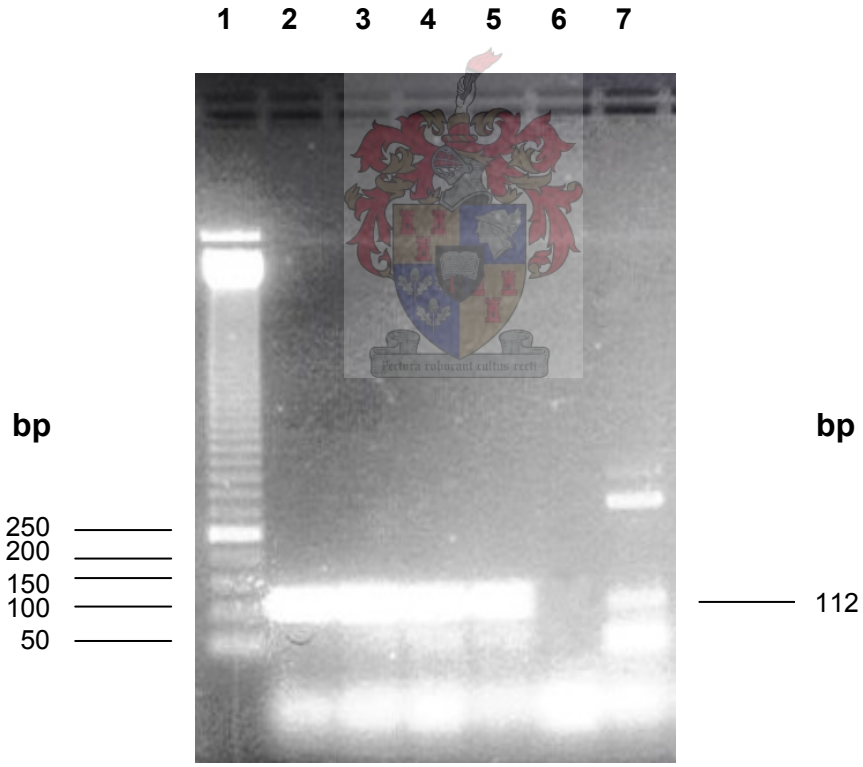


FIG. 2. DNA banding patterns obtained for strain ST4SA after amplification with genus-specific primers. Lane 1: 50-bp molecular marker from Amersham (Bioscience, UK Limited, Buckinghamshire, UK), lanes 2 to 5: Strain ST4SA, lane 6: negative control (no DNA), lane 7: positive control (*E. faecium* LMG 8149). LMG: Laboratorium voor Mikrobiologie, University of Ghent, Ghent, Belgium.

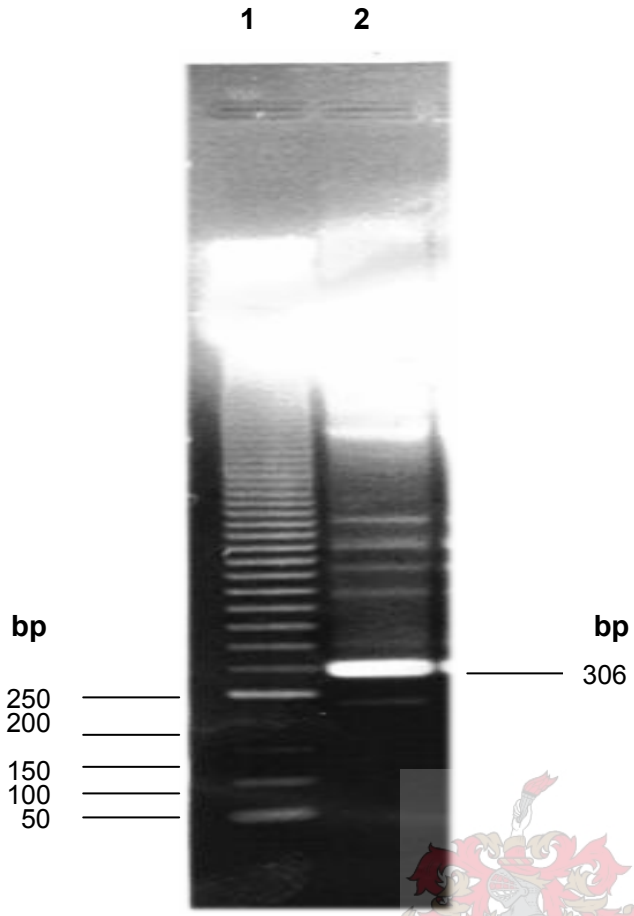


FIG. 3. DNA banding patterns obtained for strain ST4SA after amplification with species-specific primers. Lane 1: 50-bp molecular marker from Amersham (Bioscience, UK Limited, Buckinghamshire, UK), lane 2: strain ST4SA.

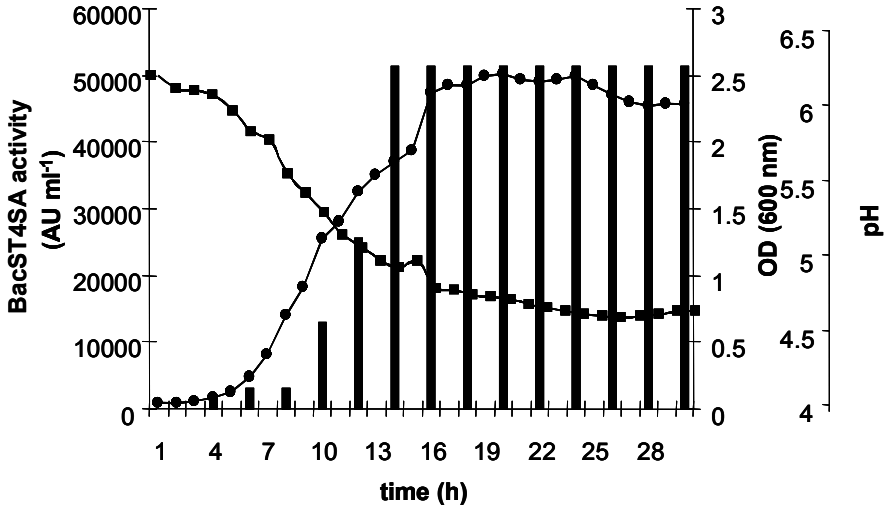


FIG. 4. Growth of strain ST4SA in MRS broth. (●) = Optical density recorded at 600nm, (■) = pH of culture, and (▌) = activity of bacST4SA, expressed as AU/ml.

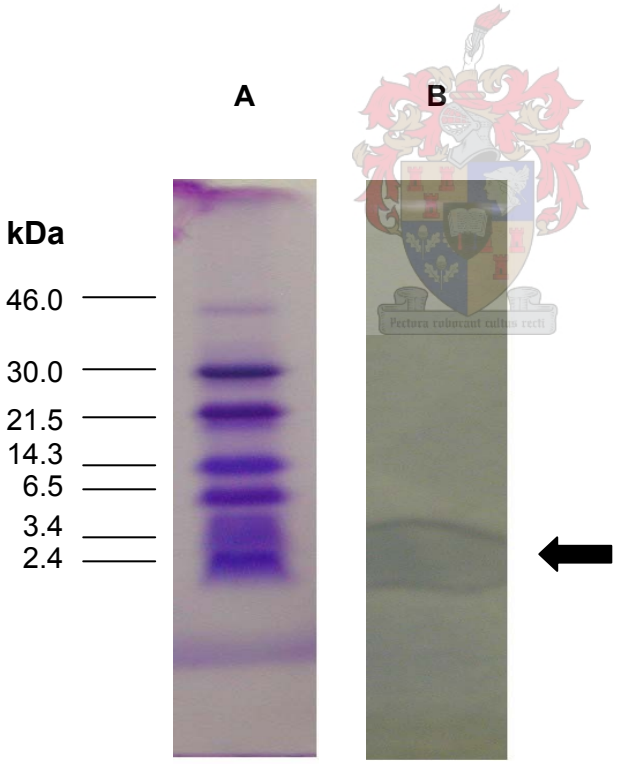
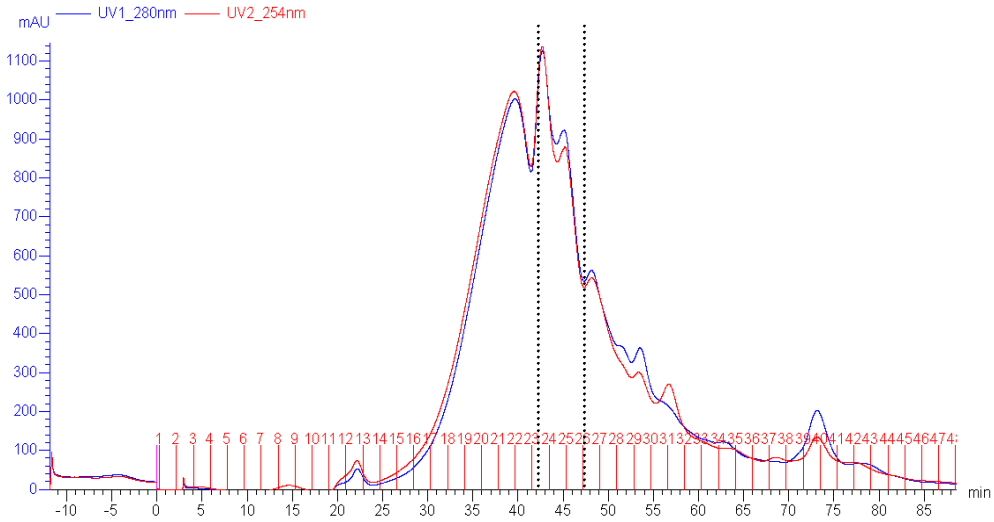


FIG. 5. Separation of bacST4SA by tricine-SDS PAGE. A: molecular weight marker from Amersham (Bioscience, UK Limited, Buckinghamshire, UK), B: bacST4SA, overlaid with *L. casei* LHS (*ca*. 10^6 cfu ml^{-1}) imbedded in MRS agar. The arrow indicates growth inhibition by bacST4SA.

A



B

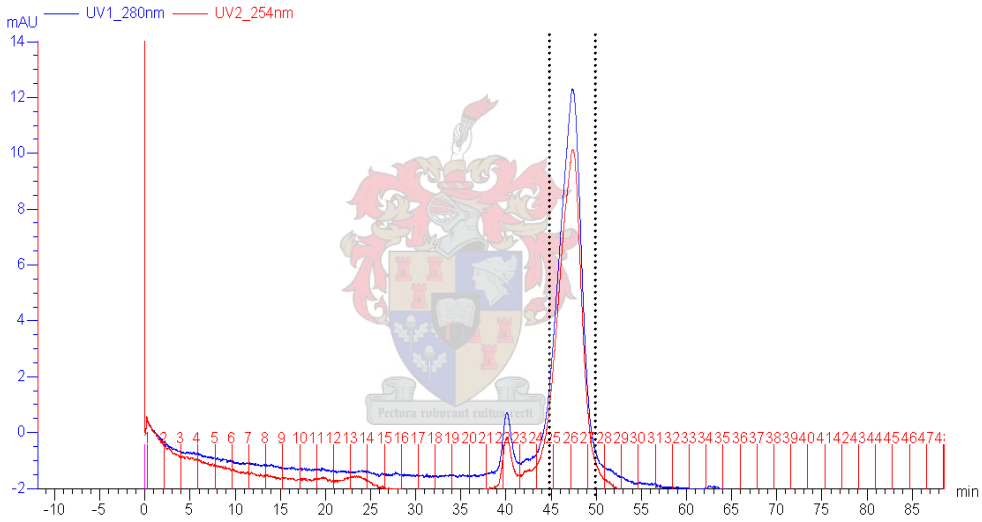


FIG. 6. Separation of bacST4SA with the ÄKTA*purifier* using a Superdex^{TM} 75 HR/10/30 column. A: First separation, B: second separation (fractions from separation A re-injected). Active peaks are indicated between dotted lines.

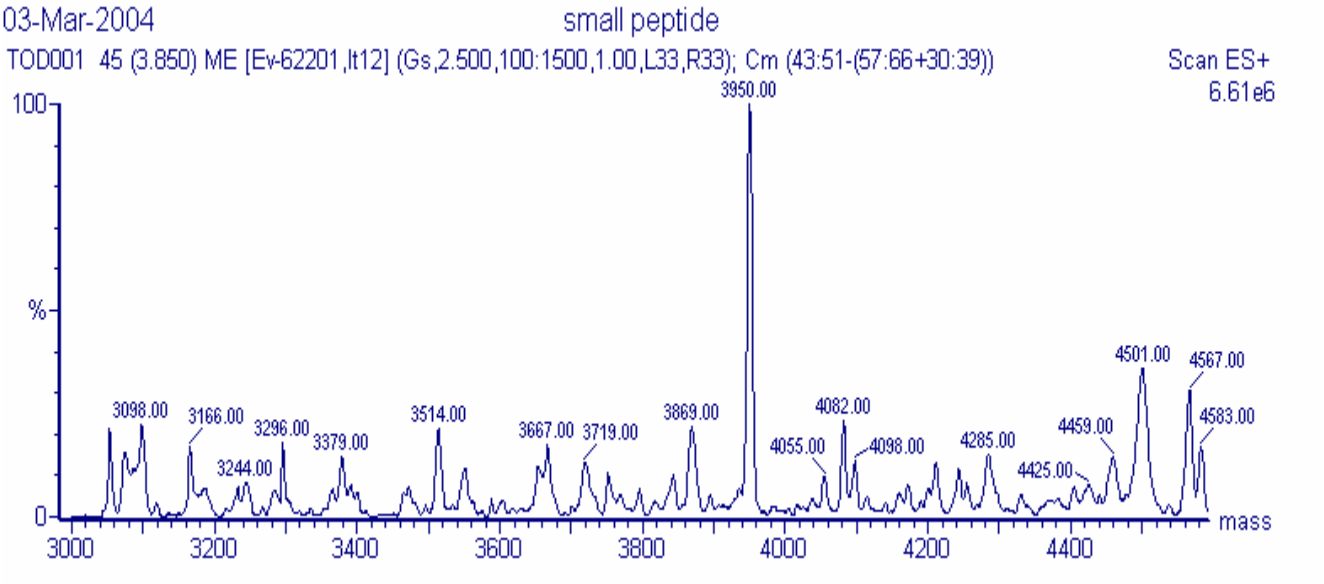


FIG. 7. Molecular mass of bacST4SA, recorded by electro-spray ionization-mass spectrometry.

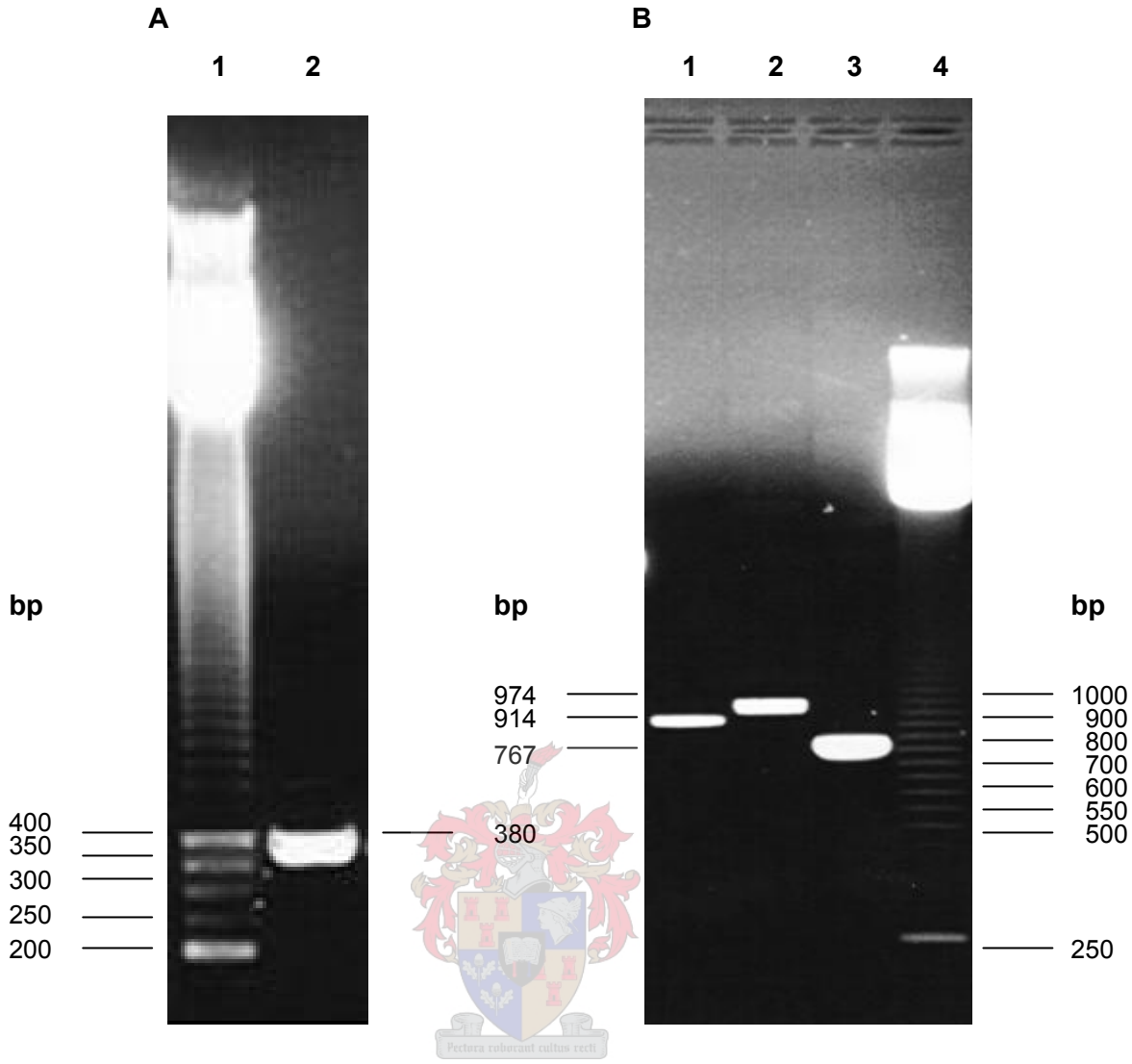


FIG. 8. DNA banding patterns obtained after amplification of the bacST4SA gene locus. A: Lane 1, 50-bp molecular marker from Amersham (Bioscience, UK Limited, Buckinghamshire, UK); lane 2, PCR amplified bacST4SA structural gene fragment (*munA*). B: Lanes 1 - 3, PCR amplified ABC-transporter$_{N\text{-terminal}}$-, ABC-transporter$_{C\text{-terminal}}$, and immunity gene (*munC*) fragments, respectively; lane 4, 50-bp molecular marker from Amersham.

A

→ Structural gene sequence

↓

Leader peptide

GTTTTGAAGAAATTAACAGCAAAAGAAATGTCACAAGTAGTAGGTGGAAAATACTACGGT
V L *K* *K* L **T** A *K* E M **S** **Q** V V G G *K* **Y** **Y** G

Mature peptide

AATGGAGTCTCATGTAATAAAAAAGGGTGCAGTGTTGATTGGGGAAAAGCTATTGGCAT
N G V **S** *C* **N** *K* *K* G *C* **S** V D **W** G *K* A I G I

TATTGGAAATAATTCTGCTGCGAATTTAGCTACTGGTGGAGCAGCTGGTTGGAAAAGTTA
I G **N** **N** **S** A A **N** L A **T** G G A A G **W** *K* **S** *

A

B

→ Immunity gene sequence

ATGAGTAATTTAAAGTGGTTTTCTGGTGGAGACGATCGACGTAAAAAAGCAGAAGTGATT
M S N L K W F S G G D D R R K K A E V I

ATTACTGAATTATTAGATGATTTAGAGATAGATCTTGGAAATTAGAGATAGAGATTAGAGA
I T E L L D D L E **I** D L G N E S L R K V

TAGATCTTGGAAATGAATCTCTTCGAAAAGTATTAGGCTCCTATCTTGAAAAGTTGAAAA
L G S Y L E **K** L K N E G T S V P L V L S

ATGAAGGAACTTCAGTTCCATTAGTTTTAAGTCGTATGAATATAGAGATATCTAATGCAAT
R M N I E I S N A I K K D G V S L N E N Q

AAAAAAAGACGGTGTATCGTTAAATGAAAATCAATCTAAAAAATTAAAAG
S K K L K E L **I** S I S N I R Y G Y

Fig. 9. Analysis of the bacST4SA gene locus. A: Amino acid sequence of the bacST4SA structural gene. Bold = polar residues; normal font = hydrophobic residues; underlined = negatively charged residues; italics = positively charged residues; ↓ = double-glycine processing site. B: The bacST4SA immunity gene. Bold = amino acid differences compared with mundticin KS.

→ ABC transporter gene sequence

```
  1       ATGCAGATGATTTTAAATAATTTTCATTCATGGATTTCAGTGGAAGTTTTAAGAGACTTA
            M  Q  M  I  L  N  N  F  H  S  W  I  S  V  E  V  L  R  D  L

 61       CTGAAACCGATTCTGAAGGTACCTGTGCATTAGGTATAGTTAACGGATTTGCTAAATTAG
 20         T  E  T  D  S  E  G  T  C  A  L  G  I  V  N  G  F  A  K  L

121       TAGGTATAGTTAACGGATTTGCTAAATTAGGAATAAATTGTGAAGCCTATAAAGCTAATA
 40       L  G  I  V  N  G  F  A  K  L  G  I  N  C  E  A  Y  K  A  N

181       GTGATGTATGGAAAGAAAATGAGTTCAATTATCCCGTAATTGCTAATATAGTAACGAATA
 60       S  D  V  W  K  E  N  E  F  N  Y  P  V  I  A  N  I  V  T  N

241       ATCAATTTCTTCATTATTGTATTGTATATGGTGTGAAAAAAGAGAAATTGTTAATAGCTG
 80       N  Q  F  L  H  Y  C  I  V  Y  G  V  K  K  E  K  L  L  I  A

301       ACCCTGCGATTGGAAAATACAAAGAATCAATAGAAAAGTTCAACAACAAATGGACTGGTG
100       D  P  A  I  G  K  Y  K  E  S  I  E  K  F  N  N  K  W  T  G

361       TTATTTTAGTTGCTGAAAAGACTCCTGATTTCCAACCCATAAATAATACAAAAAAAGTT
120       V  I  L  V  A  E  K  T  P  D  F  Q  P  I  N  N  T  K  K  S

421       TTTTTTCTTCAATAAGTTTATTAAAAGATCAATATAAAAAAATTTTATTGGTGATATTAT
140       F  F  S  S  I  S  L  L  K  D  Q  Y  K  K  I  L  L  V  I  L

481       CTTCATTAATAATAACAATTATAGGAATACTATCAAGTTACTATTTTAGAATTTTAATAG
160       S  S  L  I  I  T  I  I  G  I  L  S  S  Y  Y  F  R  I  L  I

541       ATTGGTTACTTCCTGAAAAAGACTTTTTAAATCTATTTATGATATCAATTAGCTATATCA
180       D  W  L  L  P  E  K  D  F  L  N  L  F  M  I  S  I  S  Y  I

601       TAGGCATTTTTATAACAAGTATATTTGAAATTACCAGAAGATATAATTTAGAAAAGCTAG
200       I  G  I  F  I  T  S  I  F  E  I  T  R  R  Y  N  L  E  K  L

661       GACAAGATGTAGGTAGAAGCATTTTATTTAAATATTTAGAACATATTTTCATTTTACCAG
220       G  Q  D  V  G  R  S  I  L  F  K  Y  L  E  H  I  F  I  L  P

721       CTTCCTTTTTTTCTAAAAGAAAAACTGGAGATATTGTCTCTAGATTTTCTGATGCTAATA
240       A  S  F  F  S  K  R  K  T  G  D  I  V  S  R  F  S  D  A  N

781       AAATTATAGAAGCTTTAGCTAGCTTTACTATATCTATTTTTTTAGATTTAAGTTCAGTCA
260       K  I  I  E  A  L  A  S  F  T  I  S  I  F  L  D  L  S  S  V
```

841 **TTGTTGTGGGGATTATATTGATCAATATTAATAAACAATTATTTTTAATAACGTTAAGTT**
280 **I V V G I I L I N I N K Q L F L I T L S**

901 **CTATTCCATTTTATATACTAATTATATTAGGATCAAATAAAAAAATGAGTCGATTAAACG**
300 **S I P F Y I L I I L G S N K K M S R L N**

961 **GAGAAGAGATGCAAACAAATTCAATAGTTGATTCTAATTTTATTGAAGGATTAAACGGAA**
320 **G E E M Q T N S I V D S N F I E G L N G**

1021 **TATATACTATAAAAGCACTTTGTAGTGAGAATAAGATTGTAAATCAAATATATAGAAGTT**
340 **I Y T I K A L C S E N K I V N Q I Y R S**

1081 **TAAATAAATTTTTTGATGTATCACTAAAGAGAAATATGTATGATTCTATAATTCAAAATT**
360 **L N K F F D V S L K R N M Y D S I I Q N**

1141 **TAAAAATTTTGGTTTCTCTTTTAACCTCGGCTTTTGTATTATGGCTTGGTTCGTATTATG**
380 **L K I L V S L L T S A F V L W F G S Y Y**

1201 **TTATCAATGGAGAAATTACAATAGGAGAACTAATAACTTTCAATTCAT**TATCTATATTTT
400 **V I N G E I T I G E L I T F N S** L S I F

1261 TTTCTACACCTCTACAAAATATAATAAATCTACAAGAAAAATTCCAAAAAGCACAAGTTG
420 F S T P L Q N I I N L Q E K F Q K A Q V

1321 CAAATAATCGGCTTAACGATGTATTTTCTATAAATAATGAAAATAAAGACAAGTTTATTC
440 A N N R L N D V F S I N N E N K D K F I

1381 ATT*TGGCTAAATTAACTGAAAAAGCAACGATTACATTTGAAAATGTATATTTTAGTTATT*
460 H *L A K L T E K A T I T F E N V Y F S Y*

1441 *CTACTAAATATCCTAATGTGTTAGATAATATGAGTTTTTCTCTACCTGTGAGTAAAAATA*
480 *S T K Y P N V L D N M S F S L P V S K N*

1501 *TAGGAATAAAAGGTGATAGTGGTGCTGGGAAATCAACTTTAGCACAACTTCTAGCTGGAT*
500 *I G I K G D S G A G K S T L A Q L L A G*

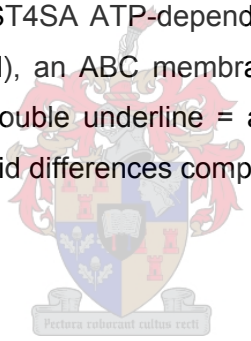
1561 *TTTACTCTCCAGATAATGGAAGAATTTGTATAAATGAGCAAAATATTGAAAATATTAATA*
520 *F Y S P D N G R I C I N E Q N I E N I N*

1621 *GAAAAGATTTACGTAAGTTGATTACCTATGTGCCTCAAGAATCTTTTATTATGAGTGGAA*
540 *R K D L R K L I T Y V P Q E S F I M S G*

1681 *CTATTAAAGACAATTTATTTTTAGGTTTAGAAAGTATTCCTGATGAACAAGAACTCGAAA*
560 *T I K D N L F L G L E S I P D E Q E L E*

```
1741    AAGTACTGAAAGATACTTGTTTATGGAGTTATATTACTGCGATTCCTCTAGGACTTGATA
580     K  V  L  K  D  T  C  L  W  S  Y  I  T  A  L  P  L  G  L  D


1801    CGTATCTAGAAGAAAATGGTGCGAATTTATCAGGTGGTCAAAAGCAAAGAATTGCTTTAG
600     T  Y  L  E  E  N  G  A  N  L  S  G  G  Q  K  Q  R  I  A  L


1861    CAAGAGTTTTATTATTAGGAAGTAAAATTTTATTATTAGATGAAGCTACGAGTGCTCTAG
620     A  R  V  L  L  L  G  S  K  I  L  L  L  D  E  A  T  S  A  L


1921    ATTCTAAAACTGAAATGCTGATTTTAGAAAAATTATTAAAGTACCCTAATAAGTCAATCA
640     D  S  K  T  E  M  L  I  L  E  K  L  L  K  Y  P  N  K  S  I


1981    TTATGATATCTCATAATGATAAATTAATAGACAAGTGTGACTTAATCATTGATTTAGACG
660     I  M  I  S  H  N  D  K  L  I  D  K  C  D  L  I  I  D  L  D


2041    AAAGGGATTCATAA
680     E  R  D  S  *
```

Fig. 10. Analysis of the bacST4SA ATP-dependent transporter gene consisting of a peptidase C39B domain (underlined), an ABC membrane-linked transporter domain (bold) and an ABC-DLP domain (italic). Double underline = amino acid differences compared to mundticin KS; dotted line = amino acid differences compared to enterocin CRL35.



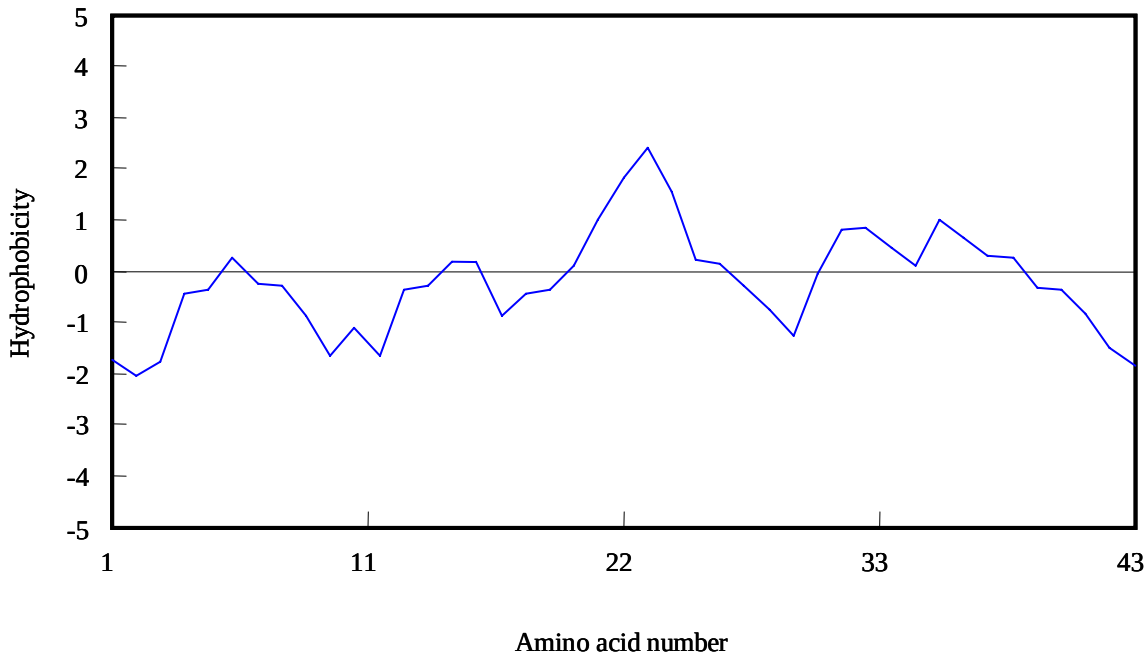


Fig. 11. Hydrophobicity profile of the bacST4SA mature peptide.

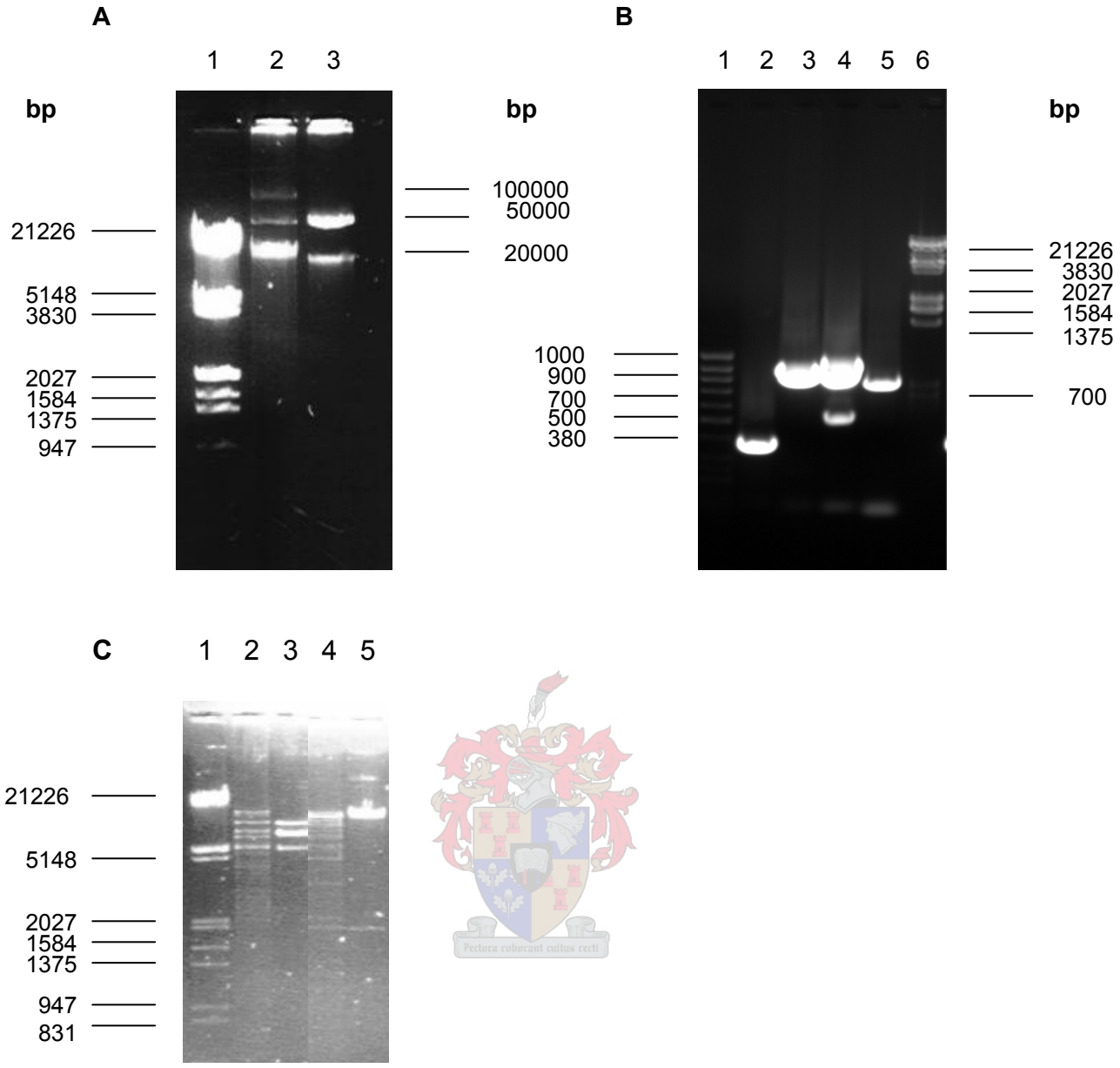


FIG. 12. Location of the bacST4SA locus on the 50-kb plasmid. A: Plasmid profile of strain ST4SA and recombinant plasmid DNA. Lane 1: lambda$_{ecoRi/HindIII}$ marker (Bioscience, UK Limited, Buckinghamshire, UK), lane 2: plasmids of strain ST4SA, lane 3: recombinant plasmid profile. B: PCR banding patterns of recombinant plasmid DNA using primers for location of the bacST4SA operon. Lane 1: 50-bp molecular marker from Amersham, lanes 2–5: PCR amplified structural gene, ABC-transporter$_{N\text{-terminal}}$, ABC-transporter$_{C\text{-terminal}}$, and immunity gene fragments, respectively, lane 6: lambda$_{ecoRi/HindIII}$ marker from Amersham. C: Restriction analysis of bacST4SA plasmid DNA (pDNA) and recombinant plasmid DNA (pDNAr). Lane 1: lambda$_{EcoRi/HindIII}$ marker from Amersham, lane 2-3: pDNA and pDNAr digested with *Eco*RI, lanes 4–5: pDNA and pDNAr digested with *Eco*RV.

Bacteriocin ST4SA, a Class IIa Peptide from *Enterococcus mundtii*, inhibits bacteria associated with Otitis Media

H. KNOETZE, S.D. TODOROV, AND L.M.T. DICKS

Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa

Correspondence to: Prof. L.M.T. Dicks, Department of Microbiology, University of Stellenbosch, 7600 Stellenbosch, South Africa. Tel: +27-21-808 5849, Fax: +27-21-808 5846, e-mail: lmtd@sun.ac.za

ABSTRACT

Bacteriocin ST4SA (bacST4SA) is a class IIa antimicrobial peptide, produced by *Enterococcus mundtii* ST4SA that has been isolated from soya beans. BacST4SA inhibits the growth of *Acinetobacter baumannii*, *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus pneumoniae,* and unidentified Gram-positive middle ear isolates A, BW, DW, F, G, and H. BacST4SA was active against *Pseudomonas aeruginosa* G, BG, I, J, B and E, although some resistance was observed. The peptide adsorbed maximally (94%) to *S. pneumoniae* 40, *P. aeruginosa* 25 and *E. faecium* HKLHS. Low concentrations of bacST4SA (51 200 AU/ml) caused DNA- and enzyme-leakage to target cells, whilst higher bacST4SA concentrations (1 638 400 AU/ml) caused cell lyses. Structural damage to cells of *E. faecium* HKLHS treated with bacST4SA was observed by atomic force microscopy. BacST4SA is bactericidal towards *E. faecium* HKLHS and bacteriostatic towards *S. pneumoniae* 40 and middle ear isolates F, BW and H. The peptide is stable between -20°C and 25°C. No decrease in antimicrobial activity was observed when tested on solid medium with human blood as a base. Maximal bacST4SA production (102 400 AU/ml) was recorded in MRS broth supplemented with tryptone, yeast extract, or a combination of tryptone and yeast extract; K_2HPO_4 (10.0 or 20.0 g/l); DL-6,8-thioctic acid; L-ascorbic acid; or thiamine. BacST4SA revealed a similar level of activity when compared with tetracycline (30µg), but revealed much higher activity compared to nasal sprays, aminoglycosides, cephalosporins, fluoroquinolones, lincosamides, macrolides, nitroimidazole, penicillin, quinolones, sulfonamides, chloramphenicol, furanzolidone, fusidic acid, rifampicin, trimethoprim, trimethoprim-sulfamethoxazole and vancomycin when tested *in vitro*.

Otitis media is one of the most common diseases diagnosed in children under the age of two (1). Pathogens associated with the disease are β-lactamase-producing *Haemophilus influenzae*, *Moraxella catarrhalis*, multi-drug resistant (MDR) *Streptococcus pneumoniae*, group A streptococci, *Staphylococcus aureus*, *Streptococcus pyogenes* and Gram-negative rods, such as *Pseudomonas aeruginosa* (1, 2). Infection is treated with amoxicillin (1-3). Higher dosages of amoxicillin, amoxicillin-clavulanate (augmentin), cefuroxime axetil and intramuscular ceftriaxone are used to control β-lactamase–producing pathogens and MDR pneumococci (1, 3). Although broad-range antibiotics are effective against a variety of Gram-positive and Gram-negative bacteria, they also affect commensial microflora and should not be used on a routine basis (4-6). When used in cases of recurrent acute otitis media, changes in selecting antibiotic resistant strains increase (3, 7-10).

In more recent papers, ribosomal synthesized peptides, known as bacteriocins, have been considered as an alternative to antibiotics (4, 6, 11-13). Bacteriocins are classified in four classes. Class I are small (<5 kDa), cationic, heat-resistant peptides, consisting of unusual amino acids, lanthionine and β-methyl-lantionine (14). Class II are small (<10 kDa), heat-stable, cationic and hydrophobic peptides (15-18). Class IIa or pediocin-like bacteriocins are characterised by the presence of an unique highly conserved N-terminal amino acid sequence, YGNGVXaaC (Tyr-Gly-Asn-Gly-Val- Xaa-Cys) consensus motif, nonpolar and small amino acids, with one or more disulphide bridges, and inhibitory to *Listeria* spp.(16, 19, 20). Two-peptide bacteriocins with separate functioning are grouped in class IIb (18, 20). Class IIc bacteriocins are thiol-activated and *sec*-dependent secreted miscellaneous peptides that requires a reduced cysteine residue to be active (17, 20, 21). Class III bacteriocins are large, hydrophilic (>30 kDa), heat-labile peptides (14, 22). Complex peptides associated with lipids or carbohydrates are grouped in class IV (14). Bacteriocins are commonly used as food preservatives (4, 23, 24). Some of these peptides have a relatively narrow spectrum of activity and inhibits closely related species by interacting with specific receptors on the target cell (6, 25). In medicine, it may be used as an antibiotic compliment, working synergistically with other antibiotic peptides, and as an antiviral agent (26-29).

Bacteriocins of class IIa are generally active against *Listeria* spp. A few have a broader spectrum of activity and inhibit *Enterococcus, Lactobacillus* and *Clostridium* spp. (24, 25, 30). The class IIa bacteriocins have a highly concerved N-terminal YGNGV consensus that binds to target cells through electrostatic interactions (20, 31). The amphiphilic or hydrophobic C-domain anchors to the hydrophobic core of the cytoplasmic membrane, causing leakage of intracellular compounds (20, 32). Class II bacteriocins normally inhibit target bacteria by dissipation of the proton motif force (PMF), including dissipation of the transmembrane potential and changes in cellular pH (20-22).

Bacteriocin production is frequently regulated by pH and growth temperature, as shown for bacteriocin ST311LD (33), enterocin 1146 (34), enterocin AS-48 (35), a bacteriocin produced by *Enterococcus faecium* RZS C5 (36), and enterocin P (37). In specific cases, higher levels of bacteriocin production have been recorded at optimal growth conditions (34, 38-42). However, apart from studies on the effect of carbon and nitrogen sources on production of enterocin P (37), bacteriocin ST311LD (33), and a bacteriocin produced by *E. faecium* CRL 1385 (42), little is known about growth conditions required for optimal bacteriocin production.

Bacteriocin ST4SA (bacST4SA) is a positively charged, hydrophobic, 43-amino acid class IIa peptide. The aim of this study was to determine the mode of activity of bacST4SA and evaluate the peptide as an alternative to antibiotics in the treatment of otitis media.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Enterococcus mundtii* ST4SA was cultured in MRS broth (Biolab, Biolab Diagnostics, Midrand, SA). The target organisms, their growth media and growth conditions are listed in Table 1.

Production and partial purification of bacST4SA. Ten ml of an 18-h-old culture of *E. mundtii* ST4SA was inoculated into 500 ml MRS broth (Biolab) and incubated at 30 °C for 20 h, without agitation. The cells were harvested (8000 x g, 10 min, 4 °C), the pH of the cell-free supernatant adjusted to 6.5 using 1 N NaOH, and treated for 10 min at 80 ºC. The peptide was precipitated from the cell-free supernatant with 80% saturated ammonium sulphate (43). The precipitate was resuspended in sterile distilled water (one-fifth of the original volume) and desalted against sterile distilled water for 18 h at 4 °C. A dialysis bag with a pore size of 1000 Da (Spectrum inc., CA, USA) was used. The pH of the dialysate (crude-extract) was adjusted to 6.5 with sterile 1 N NaOH and treated for 10 min at 80 °C. Three ml aliquots of the suspension was lyophilised and stored at -20 °C. Just before an experiment, the lyophilised crude-extract was resuspended in 0.1 ml sterile MilliQ water (Millipore, MA, USA) and treated for 10 min at 100 °C. Resuspended samples were stored at 4 °C. Antimicrobial activity of bacST4SA was determined by using the agar-spot test method described by Ivanova *et al.* (44). Activity was expressed in arbitrary units (AU) per ml. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (44).

Adsorption of bacST4SA. Adsorption of bacST4SA to *S. pneumoniae* 27, 29, 40 and D, *Enterococcus faecalis* 20 and 21, *P. aeruginosa* E, J, I and 25, *S. aureus* 13, middle ear isolates BW, DW, F, H and G, and *E. faecium* HKLHS were studied as described by Todorov and Dicks (45). Target strains were grown to mid-log phase, harvested (10 000 x g, 15 min, 4 °C), washed twice with sterile 5 mM phosphate buffer (pH 6.5), and re-suspended to the original volume in the same buffer. Cell suspensions (0.7 ml) were mixed with an equal volume of bacST4SA (819 200 AU/ml). Samples were incubated at 37 °C for 1 h, the cells harvested (10 000 x g, 15 min, 4 °C), and the activity of unbound bacST4SA determined as described before. Resuspended cell suspensions, mixed with an equal volume of MRS medium, without bacST4SA, were used as controls. All experiments were conducted in duplicate. Adsorption of bacST4SA to target cells was calculated according to the following formula:

% Adsorption = 100 – [(bacteriocin activity after treatment/ original bacteriocin activity) x 100]

Mode of action. Target strains (Table 2) were cultured overnight as described before. Cells were harvested (10 000 x g, 15 min, 4 °C) and washed twice with sterile 5 mM phosphate buffer (pH 6.5). Cell-free supernatant containing bacST4SA was added to washed target cells at a final concentration of 5 160 AU/ml and incubated at 37 °C for 1 h. Cells were harvested (10 000 x g, 15 min, 4 °C) and the supernatant filtered through a 0.2 µm nitrocellulose membrane (Minisart®, Sartotius). Changes in external levels were recorded with optical density readings at 260 nm. The controls were target cells suspended in 5 mM phosphate buffer and not treated with bacST4SA.

In a separate experiment, extracellular levels of β-galactosidase were monitored: Ten ml cultures of target strains (12-h-old) were harvested (10 000 x g, 15 min, 4 °C), the cells washed with 0.03 M sodium phosphate buffer (pH 6.5) and re-suspended in 2 ml of the same buffer. To each cell suspension, 2 ml bacST4SA cell-free supernatant was added to a final concentration of 25 600 AU/ml. After 5 min at 25 °C, 0.2 ml 0.1 M ONPG (O-nitrophenyl-β-D-galactopyranosidase) in 0.03 M sodium phosphate buffer (pH 6.8) was added and incubated at 37 °C for 10 min. The enzymatic reaction of β-galactosidase was stopped by adding 2.0 ml 0.1 M sodium carbonate. Cells were harvested (10 000 x g, 15 min, 25 °C) and optical density readings recorded at 420 nm. Controls were cells prepared in the same way, but not treated with bacST4SA. All experiments were performed in duplicate.

In another experiment, *E. faecium* HKLHS and *S. pneumoniae* 40 were cultured overnight in MRS and BHI media, respectively. *E. faecium* HKLHS was inoculated (2.0%, v/v) into 100 ml MRS broth (Biolab) and incubated at 30 °C. *S. pneumoniae* 40 was

inoculated (0.2%, v/v) into 100 ml BHI medium (Merck, Darmstadt, Germany) and incubated at 37 °C. Ten ml bacST4SA (1 638 400 AU/ml) was added to both cultures at mid-exponential growth and incubated for a further 12 h. Optical density readings (600 nm) were recorded every hour. The same experiment was performed with unidentified strains BW, F and H isolated from patients with acute otitis media infections.

The effect of bacST4SA on cell morphology was determined by atomic force microscopy, as described by Todorov *et al.* (46). BacST4SA (49 152 AU/ml, final concentration) was added to an 18-h-old cell suspension and incubated for 1 h at 30 °C. Cells were then harvested (5000 x g, 10 min, 4 °C) and washed five times with 10 ml sterile distilled water before subjected to microscopy.

Effect of growth conditions and medium compounds on bacST4SA production and stability. *E. mundtii* ST4SA was grown in 10 ml MRS broth (Biolab) for 18 h at 30 °C. Cells were harvested (10 000 x g, 10 min, 4 °C) and the pellet resuspended in 10 ml sterile physiological salt. Four ml of this suspension was used to inoculate 200 ml of the following media: (i) MRS broth (47), without organic substrates, supplemented with 1.0 to 40.0 g/l tryptone, 20.0 g/l meat extract, 20.0 g/l yeast extract, 12.5 g/l tryptone plus 7.5 g/l yeast extract, 10.0 g/l meat extract plus 10.0 g/l yeast extract, and 12.5 g/l tryptone plus 7.5 g/l meat extract, respectively; (ii) MRS broth (47) with 20.0 g/l glucose; (iii) MRS broth (47) without glucose, supplemented with 20.0 g/l maltose, mannose, fructose, lactose and saccharose, respectively; (iv) MRS broth (47) with 2.0 g/l KH_2PO_4 and 2.0 - 50.0 g/l K_2HPO_4; respectively (the pH was corrected to 6.5 with 0.1 M HCl); (v) MRS broth (47) with 0.0 to 10.0 g/l tri-ammonium sulphate; (vi) MRS broth (47) with 0.0 to 0.5 g/l magnesium sulphate; (vii) MRS broth (47) with 0.0 to 0.5 g/l manganese sulphate; (viii) MRS broth (47) with 0.0 to 10.0 g/l sodium acetate; and (ix) MRS broth (47) supplemented with 1.0 to 50.0 g/l glycerol. Antimicrobial activity was determined using the agar-spot test method as described before.

MRS broth (47) was supplemented with filter-sterilized cyanocobalamin (Sigma, St. Louis, Mo.), L-ascorbic acid (BDH Chemicals, Ltd, Poole, UK), thiamine (Sigma), phylloquinone (Sigma) and DL-6,8-thioctic acid (Sigma) to a final concentration of 1.0 mg/l. Incubation was at 30 °C for 20 h. Antimicrobial activity was determined using the agar-spot test method as described before. MRS broth (47) served as control.

In another experiment, *E. mundtii* ST4SA was grown in 10 ml LAPTg broth (48) and incubated for 18 h at 30 ºC. The cells were harvested a resuspended as described before. Four ml of this suspension was used to inoculate 200 ml of the following media: (i) LAPTg broth (48), without organic nutrients, supplemented with 35.0 g/l peptone from casein, 35.0 g/l yeast extract and 35.0 g/l tryptone, respectively; (ii) LAPTg broth (48) with 5.0 to 20.0 g/l

glucose; and (iii) LAPTg broth (48) without Tween 80. Activity levels were determined using the agar-spot test method. LAPTg broth (48) served as control.

BacST4SA was suspended in middle ear fluid and blood (819 200 AU/ml, final concentration) and changes in activity determined by using the spot-on-lawn method. Samples were tested after 1, 2, 6 and 12 h at 37 °C. In a separate experiment, bacST4SA was suspended in sterile distilled water and incubated at -20 °C, 0 °C, 25 °C, 37 °C and 60 °C, respectively, for 6 weeks. Activity was recorded every seven days, as described before.

Comparison of bacST4SA with antibiotics and ototic drop suspensions. *E. faecium* HKLHS and middle ear pathogens, *S. pneumoniae* 27 and *P. aeruginosa* J, were grown for 12 h in MRS broth (Biolab) and BHI broth (Merck), respectively. MRS pour-plates (Biolab) were prepared, containing 5% (v/v) *E. faecium* HKLHS (*ca.* 10^7 cfu/ml). BHI pour-plates (Merck), contained 5% (v/v) *S. pneumoniae* 27 (*ca.* 10^8 cfu/ml) and *P. aeruginosa* J (*ca.* 10^8 cfu/ml), respectively. An antibiotic disc (Table 4) and10 µl bacST4SA (1 638 400 AU/ml) were dropped onto the surface of the inoculated pour-plates and incubated for 18 h at 37 °C. Activity was compared by measuring the diameter of the inhibition zones.

RESULTS

All data represents an average of three repeats. The optical density values recorded in each experiment did not vary by more than 5% and standard deviation values are not shown. In case of bacteriocin production (AU/ml), all three repeats produced the same result.

Isolation of bacST4SA. The cell-free supernatant of an 18-h-old culture of strain ST4SA yielded bacST4SA levels of 25 600 AU/ml. The activity after ammonium sulphate precipitation increased to 819 200 AU/ml, but decreased to 204 800 AU/ml after dialysis. Lyophilisation, followed by resuspension into smaller volumes increased the activity to 1 638 400 AU/ml, i.e. 64-fold higher than in the culture supernatant.

Spectrum of antimicrobial activity. BacST4SA inhibited the growth of *Acinetobacter baumannii* 16, *E. faecalis* 20 and 21, *E. faecium* HKLHS, *S. aureus* 36, *S. pneumoniae* 27, 29 and 40, and unknown middle ear isolates A, BW, DW, F, G, and H (Table 1). No activity was observed against *A. baumannii* 19, *H. influenzae* C, *P. aeruginosa* 8, 14, and 25, *S. aureus* 13, *Staphylococcus carnosus* LMG 13567 and *S. pneumoniae* A, D, and 10 (Table 1). BacST4SA was active against *P. aeruginosa* G, BG, I, J, B and E, although resistance (opaque zones) was observed for some strains (Table 1).

Adsorption of bacST4SA to target strains. BacST4SA adsorbed at different levels to target strains. Maximum adsorption (94%) was recorded to cells of *S. pneumoniae* 40, *P. aeruginosa* 25 and *E. faecium* HKLHS. A smaller quantity (75%) of the peptide adsorbed to *S. pneumoniae* 27, 29 and D, *E. faecalis* 20 and 21, *P. aeruginosa* I and J, *S. aureus* 13 and unidentified middle ear isolates BW, DW, F, H and G and only 50% adsorbed to *P. aeruginosa* E.

Mode of action. Treatment of pathogens with bacST4SA (25 600 AU/ml) led to the leakage of cellular components (DNA, RNA, proteins, etc.) and β-galactosidase (Table 2). Cells treated with bacST4SA showed a clear difference in cell structure (Fig. 1). The surface of *E. faecium* HKLHS treated with bacST4SA changed from smooth (Fig. 1A) to rough (Fig. 1B) after 1 h in contact with bacST4SA. Leakage of intracellular compounds from damaged cells was clearly visible (Fig. 1C and D).

Addition of bacST4SA to *S. pneumoniae* 40 (Fig. 2A), middle ear isolates F (Fig. 2B) and BW (Fig. 2C), and *E. faecium* HKLHS (Fig. 2E) led to a rapid decline in cell density. Isolate H was less sensitive to bacST4SA and started to recover after the first hour of treatment (Fig. 2D). This was, however, followed by a decrease in cell density after 2 h. The growth of *E. faecium* HKLHS was completely inhibited (Fig. 2E).

Effect of medium compounds and vitamins on bacST4SA production. The effect of medium compounds on bacST4SA production is shown in Table 3. Optimal production of bacST4SA (51 200 AU/ml) was recorded in MRS broth with glucose, saccharose or maltose as sole carbon source. Approximately half the activity (25 600 AU/ml) was recorded when cells were grown in the presence of mannose and lactose (Table 3). Growth in MRS broth with 20.0 g/l meat extract, 15.0 g/l tryptone, a combination of 10.0 g/l meat extract and 10.0 g/l yeast extract, 12.5 g/l tryptone and 7.5 g/l meat extract yielded 51 200 AU/ml. The activity doubled (102 400 AU/ml) when cells were grown in MRS broth supplemented with 20.0 g/l tryptone, 20.0 g/l yeast extract, a combination of 10.0 g/l yeast extract and 10.0 g/l tryptone, or a combination of 12.5 g/l tryptone and 7.5 g/l yeast extract as sole nitrogen source. Growth in MRS broth without nitrogen sources led to a 98% decrease in bacST4SA production.

Relatively good growth of bacST4SA was observed in MRS broth with different concentrations of tri-ammonium citrate, magnesium sulphate, manganese sulphate and sodium acetate. Production of bacST4SA was affected by different concentrations. The absence or 0.5, 1.0, and 5.0 g/l tri-ammonium citrate resulted in a 75% reduction of bacST4SA production (Table 3). Growth in the presence of 10.0 g/l tri-ammonium citrate led

to a 50% decrease in bacST4SA production (Table 3). Maximum bacST4SA production (51 200.AU/ml) was recorded in the presence of 0.1 g/l magnesium sulphate. In the absence of magnesium sulphate, or at higher concentrations, a decrease in bacST4SA production was observed. However, 1.0 g/l magnesium sulphate led to a 98% decrease in bacST4SA production (Table 3). Similar results have been recorded for manganese sulphate, with optimal activity (51 200 AU/ml) obtained in the presence of 0.05 g/l. Increased concentrations of manganese sulphate (0.2 and 0.5 g/l) led to 87.5% reduction in bacST4SA production (Table 3). The optimal concentration of sodium acetate for bacST4SA production was 5.0 g/l. A higher concentration (10.0 g/l) decreased bacST4SA production by 50% (Table 3). Growth in MRS without sodium acetate or in the presence of 1.0 g/l decreased bacST4SA production by 87.5% (Table 3). Higher levels of K_2HPO_4 (10.0 and 20.0 g/l) yielded 102 400 AU/ml bacST4SA (Table 3). Growth in the presence of 50.0 g/l K_2HPO_4 decreased bacST4SA production by 87.5%. Replacement of K_2HPO_4 with KH_2PO_4 resulted in a 50% decrease in bacST4SA production (Table 3).

No change in bacST4SA production was recorded when cells were grown in medium without glycerol and in the presence of 1.0 g/l. BacST4SA activity was inhibited when cultured in MRS broth enriched with glycerol higher than 1.0 g/l. Production of bacST4SA changed to 12 800 AU/ml when MRS broth was supplemented with 20.0 g/l and 50.0 g/l glycerol.

A high level of bacST4SA (102 400 AU/ml) was produced when cells were grown in MRS broth enriched with L-ascorbic acid, thiamine and DL-6,8-thioctic acid. No change in activity was observed when cells were grown in the presence of cyanocobalamin. However, the addition of phylloquinone to MRS broth led to a decrease in bacST4SA activity (25 600 AU/ml).

Optimal bacST4SA production (12 600 AU/ml) was recorded in the presence of LAPTg broth supplemented with 5.0 g/l glucose. However, bacST4SA production decreased by 50% when cultured in the presence of 35.0 g/l peptone, 35.0 g/l tryptone, or 35.0 g/l yeast extract, glucose (15.0 or 20.0 g/l), or in the absence of Tween 80.

Effect of growth conditions on bacST4SA stability. BacST4SA remained active after 12 h of incubation in the presence of blood and 6 h in the presence of middle ear fluid. A two-fold decrease in activity was recorded after 12 h in the presence of middle ear fluid (not shown).

BacST4SA was stable at -20 °C for at least 6 weeks and 3 weeks at 4 °C, with a two-fold decrease after 4 weeks (Fig. 3). Activity continued to be stable for up to week 6. At 25 °C, bacST4SA activity decreased two-fold after one week and remained stable for the duration of the experiment (Fig. 3). At 37 °C bacST4SA remained stable for one week,

followed by a four-fold decrease. After the fourth week a two-fold decrease was observed, which remained stable for the duration of the experiment (Fig. 3). No activity was observed for bacST4SA incubated at 60 °C (Fig. 3).

Comparison of bacST4SA crude-extract with other antimicrobial agents.

BacST4SA inhibited the growth of *E. faecium* HKLHS and middle ear pathogens *P. aeruginosa* J and *S. pneumoniae* 27 (Table 4). After prolonged incubation, *P. aeruginosa* J developed resistance. Similar results were recorded for tetracycline (30 µg), CiproBay®HC otic drops, and Exocin. However, lower levels of activity were recorded against *P. aeruginosa* J, *E. faecium* HKLHS and *S. pneumoniae* 27.

Exocin was highly active against *P. aeruginosa* J and *S. pneumoniae* 27, with less activity against *E. faecium* HKLHS. Tetracycline (30 µg) inhibited *S. pneumoniae* 27 and *E. faecium* HKLHS, with less activity and some resistance against *P. aeruginosa* J. BacST4SA, Augmentin (30 µg) and compound sulphonamides (300 µg) revealed similar activity against *P. aeruginosa* J, although some resistance was observed, whereas nitrofurantoin (300 µg), cefepime (30 µg), amikacin (30 µg), Sofradex® and Tobrex® presented the same level of activity against *P. aeruginosa* J, with no resistance observed. Ciprofloxacin (5 µg) had the best activity against *P. aeruginosa* J. However, resistance was recorded.

S. pneumoniae 27 was inhibited by bacST4SA, Exocin, amoxicillin (10 µg), Augmentin (30 µg) and tetracycline (30 µg) and revealed the highest activity against this strain. The other antibiotics had little or no activity against *S. pneumoniae* 27.

BacST4SA, CiproBay®HC otic, Exocin, Flomist, Vibrocil, amoxicillin (10 µg), Augmentin (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), chloramphenicol (10 µg), ciprofloxacin (5 µg), erythromycin (5 µg), furazolidone (50 µg), nitrofurantoin (300 µg), oflaxacin (5 µg), rifampicin (5 µg) and tetracycline (30 µg) were active against all three pathogens. However, Flomist, Vibrocil, cefotaxime (30 µg), ceftriaxone (30 µg), erythromycin (5 µg), furazolidone (50 µg), oflaxacin (5 µg), and rifampicin (5 µg) revealed very low activity. Amoxicillin (10 µg), Augmentin (30 µg) and nitrofurantoin (300 µg) had low activity against *E. faecium* HKLHS. Slight antibiotic resistance to Augmentin (30 µg) was detected for *P. aeruginosa* J. Chloramphenicol (10 µg) revealed very low activity against *S. pneumoniae* 27 with some resistance against *P. aeruginosa* J. Ciprofloxacin (5 µg) inhibited *S. pneumoniae* 27 and *E. faecium* HKLHS. Cefuroxime (30 µg), metronidazole (5 µg), oxacillin (1 µg), sulfafurazole (25 µg), sulfamethoxazole (25 µg), and trimethoprim (25 µg) did not inhibit the growth of *E. faecium* HKLHS and middle ear pathogens *P. aeruginosa* J and *S. pneumoniae* 27.

DISCUSSION

BacST4SA is active against *E. faecalis* 20 and 21, *E. faecium* HKLHS, *S. aureus* 36, *S. pneumoniae* 27, 29 and 40, and unidentified middle ear isolates A, BW, DW, F, G, and H, and Gram-negative bacteria *A. baumannii* 16 and *P. aeruginosa* J. By definition, bacteriocins are only inhibitory towards closely related species (6). The activity against Gram-negative bacteria is thus an unusual phenomenon and has been reported for only a few bacteriocins. Enterocin AS-48, a class IIc bacteriocin produced by *E. faecalis* S-48, is active against a variety of Gram-negative bacteria (49). Enterocin 012, produced by *Enterococcus gallinarum,* inhibits the growth *P. aeruginosa* and *Escherichia coli* (50), whilst enterocin MR99 of *E. faecalis* inhibits *E. coli*, but not *P. aeruginosa* (51). Activity against *A. baumannii* has not been reported for other bacteriocins produced by the genus *Enterococcus.* However, a bacteriocin produced by *Lactobacillus plantarum* isolated from molasses, revealed activity against *A. baumannii* (52). BacST4SA is the only class IIa bacteriocin active against *S. pneumoniae* and other Gram-positive middle ear isolates. The class IIc enterocins, 50LA and 50LB produced by *Enterococcus faecium* L50, are active against *S. pneumoniae* (53). Only certain strains of *S. pneumoniae* were inhibited by bacST4SA. It might be that bacST4SA activity is serotype specific. In general, it is difficult to compare the spectra of activity of different class IIa bacteriocins, since the target strains, level of purification and methods used in antibacterial assays differ.

BacST4SA adsorbed to sensitive and resistant cells of Gram-positive and Gram-negative bacteria. Adsorption ranged from 50% for *P. aeruginosa* E to 94% for *S. pneumoniae* 40, *P. aeruginosa* 25 and *E. faecium* HKLHS. No differences were detected in adsorption levels between sensitive and resistant strains. Similar findings have been reported by other authors. Buhnericin LB, produced by *Lactobacillus buchneri*, adsorbed 100% to *L. plantarum, Pediococcus dextranicus, Oenococcus oeni* and *E. faecalis* (54). However, the authors also reported 100% adsorption of the peptide to a resistant strain of *Pediococcus cerevisiae* LB (54). Similar results have been recorded by Manca de Nadra *et al.* (55). Pediocin N5p adsorbed at higher levels to sensitive strains *O. oeni* X2L, *Lactobacillus hilgardii, O. oeni* L10, and *L. higardii* 6D (55). Adsorption levels below 50% were recorded for resistant bacteria. The percentage adsorption might depend on the assay, number and type of receptors available on the cytoplasmic membrane, surface area and several environmental factors.

BacST4SA is an amphiphilic and cationic peptide, which interacts with negatively charged cytoplasmic membranes of sensitive cells. In this study, low concentrations of bacST4SA (51 200 AU/ml) caused the formation of small pores and the leakage of small intracellular molecules, such as DNA and enzymes. Higher levels of bacST4SA resulted in

an immediate efflux of intracellular macromolecules, as observed by atomic force microscopy. The mode of activity is most probably dissipation of the proton motif force. Similar results were reported for enterocin CRL35 (56, 57), mundticin KS (58), mundticin AT06 (59) and enterocin P (60-62) produced by *E. mundtii* CRL 35 (formerly known as *E. faecium* CRL 35), *E. mundtii* NFRI 7393, *E. mundtii* AT06 and *E. faecium* P136. The effect of bacST4SA on *E. faecium* HKLHS was bactericidal, whereas bacST4SA was bacteriostatic towards *S. pneumoniae* 40 and middle ear isolates F, BW and H.

The activity of bacST4SA remained unchanged when incubated in the presence of blood and middle ear fluid, suggesting that the peptide could be used in the control of middle ear infections. The peptide is also relatively stable over a broad pH range (pH 2 to 12) and at high and low temperatures (6 weeks at -20 °C, 3 weeks at 4 °C and one week at 37 °C). Similar findings were reported for other enterocins. Enterocin EJ97, produced by *E. faecalis* EJ97, is stable for 6 months at -20 °C and -70 °C (63). Mundticin AT06 was stable at 4 °C for the 14 h tested (59).

Growth in the presence of tryptone and yeast extract as sole nitrogen source, or a combination of tryptone and yeast extract yielded increased levels of bacST4SA (102 400 AU/ml). Similar results were reported for plantaricin 423 produced by *L. plantarum* 423 (64) and bacteriocin ST311LD produced by *E. faecium* ST311LD (33). Substitution of glucose with saccharose or maltose had no effect on bacST4SA production. However, lactose and mannose decreased bacST4SA production by 50%. Similar results were reported for bacteriocin ST311LD in the presence of glucose and maltose (33). However, low levels (5.0 or 10.0 g/l) of saccharose increased bacteriocin ST311LD production. The effect of glucose on production of enterocin 1146 (65), plantaricin UG1 (66) and plantaricin ST31 (41) has been reported. Maximal levels of bacteriocin activity were produced by *E. faecium* RZS C5 cultured in MRS broth supplemented with lactose (5.0%, w/v), adjusted to pH 6.5, and incubated at 37 °C (67).

Little is known about the effect mineral ions have on production of bacteriocins. Different bacST4SA activity levels were recorded in the presence of K_2HPO_4 or KH_2PO_4. The highest bacST4SA production (102 400 AU/ml) was obtained when cells were grown in the presence of 10.0 or 20.0 g/l K_2HPO_4. No difference in bacteriocin ST311LD production was recorded when cells were cultured in medium supplemented with 2.0 g/l K_2HPO_4 or 2.0 g/l KH_2PO_4. Increased levels of K_2HPO_4 resulted in increased bacteriocin ST311LD production (33).

Different concentrations of tri-ammonium citrate, magnesium sulphate, manganese sulphate, and sodium acetate influenced the production of bacST4SA. Exclusion of these components in MRS broth decreased the level of bacST4SA production. Optimal bacST4SA production (51 200 AU/ml) was recorded in the presence of 2.0 g/l tri-ammonium citrate, 0.1

g/l magnesium sulphate, 0.05 g/l manganese sulphate, or 5.0 g/l sodium acetate. MRS broth contains 2.0 g/l tri-ammonium citrate, 0.1 g/l magnesium sulphate, 0.05 g/l manganese sulphate, and 5.0 g/l sodium acetate (47). Higher or lower concentrations of these compounds led to decreased activity. Changes in the levels of manganese sulphate and magnesium sulphate did not influence the production of bacteriocin QU 2 (68). The effect of cations and anions on bacteriocin production may be strain specific (69).

Optimal levels of bacST4SA production (51 200 AU/ml) were recorded in the presence of 1.0 g/l glycerol. Glycerol concentrations higher and lower than 1.0 g/l resulted in the inhibition of activity. Similar results were reported for bacteriocin ST311LD (33) and plantaricin ST31 (41). Glycerol concentrations higher than 1.0 g/l decreased bacteriocin production, probably due to changes in osmotic stress.

Production of bacST4SA is stimulated in the presence of Tween 80. Similar results were obtained for bacteriocin QU 2 (68). Tween 80 had no effect on the production of enterocin EJ97 (63). However, it decreased the production of enterocin MR99 (51). Tween 80 may stimulate bacteriocin production by decreasing the absorption of the bacteriocin to the producer strain, glass and polypropylene surfaces (69-71).

The use of cationic peptides as a new alternative for treatment of various diseases has become of major interest. BacST4SA is more active towards middle ear pathogens, compared to other antimicrobial agents. This suggests that bacST4SA may be used as an alternative in the treatment of otitis media. Aminoglycosides, fluoroquinolons, quinolones, lincosamides, macrolides, rifampicin, glycopeptides, vancomycin, tetracycline, chloramphenicol and several other antibiotics cause intracellular damage and require specific target molecules as receptors to initiate growth inhibition (72). Penicillin and cephalosporin antibiotics inhibit peptidoglycan cross-linkages, thus preventing cell wall synthesis (72). Bacteriocins only require certain docking molecules to initiate growth inhibition (4).

BacST4SA revealed similar, and in some cases better, activity when compared to other antimicrobial agents. However, for bacST4SA to be accepted in otitis media treatment a number of clinical tests will have to be done. Currently, the toxicity, route of administration and pharmacological profile of bacST4SA are being investigated.

ACKNOWLEDGEMENT

This research was funded by Cipla Medpro (Pty) Ltd and the National Research Foundation (NRF), South Africa.

REFERENCES

1. **Segal, N., E. Leibovitz, R. Dagan, and A. Lieberman.** 2005. Acute otitis media diagnosis and treatment in the era of antibiotic resistant bacteria: updated clinical practice guidelines. Int. J. Otorhinolaryngol. **69:**1311-1319.

2. **McCracken, G. H.** 2002. Diagnosis and management of acute otitis media in the urgent care setting. Ann. Emerg. Med. **39:**413-421.

3. **Klein, J. O.** 1999. Management of acute otitis media in an era of increasing antibiotic resistance. Int. J. Pediatr. Otorhinolaryngol. **49(1):**S15-S17.

4. **Cleveland, J., T. J. Montville, I. F. Nes, and M. L. Chikindas.** 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. **71:**1-20.

5. **Brook, I., and A. E. Gober.** 1998. Bacterial interference in the nasopharynx following antimicrobial therapy of acute otitis media. J. Antimicrob. Chemother. **41:**489-492.

6. **Riley, M. A., and J. E. Wertz.** 2002. Bacteriocins: evolution, ecology, and application. Annu. Rev. Microbiol. **56:**117-137.

7. **Lisby-Sutch, S., M. A. Nemec-Dwyer, R. G. Deeter, and S. M. Gaur.** 1990. Therapy of otitis media. Clin. Pharmacol. **9:**15-34.

8. **Rovers, M. M., A. G. Schilder, G. A. Zielhuis, and R. M. Rosenfeld.** 2004. Otitis media. Lancet. **363(9407):**465-473.

9. **Block, S. L., C. L. Harrison, J. Hedrick, R. Tyler, A. Smith, and R. Hedrick.** 2001. Restricted use of antibiotic prophylaxis for recurrent acute otitis media in the era of penicillin non-susceptible *Streptococcus pneumoniae*. Int. J. Pediatr. Otorhinolaryngol. **61(1):**47-60.

10. **Faden, H.** 2001. The microbiologic and immunologic basis for recurrent otitis media in children. Eur. J. Pediatr. **160(7):**407-413.

11. **Hancock, R. E., and A. Patrzykat.** 2002. Clinical development of cationic antimicrobial peptides: from natural to novel antibiotics. Curr. Drug Targets Infect. Disord. **2:**79-83.

12. **Ryan, M. P., J. Flynn, C. Hill, R. P. Ross, and W. J. Meaney.** 1999. The natural food grade inhibitor, Lacticin 3147, reduced the incidence of mastitis after experimental challenge with *Streptococcus dysgalactiae* in non-lactating dairy cows. J. Dairy Sci. **82:**2625-2631.

13. **Delves-Broughton, J., P. Blackburn, R. J. Evans, and J. Hugenholtz.** 1996. Applications of the bacteriocin, nisin. Antonie van Leeuwenhoek **69:**193-202.

14. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:**39-86.

15. **De Vuyste, L., and E. J. Vandamme.** 1994. Antimicrobial potential of lactic acid bacteria, p. 91-142. *In* L. De Vuyst, and E. J. Vandamme (ed.), Bacteriocins of lactic acid bacteria: Microbiology, Genetics and Application, Blackie Academic and Professional, London, UK.

16. **Nes, I. F., B. D. Diep, L. S. Havarstein, M. B. Brurberg, V. Eijsink, and H. Holo.** 1996. Biosynthesis of bacteriocins of lactic acid bacteria. Antonie van Leeuwenhoek **70:**113-128.

17. **Abee, T., L. Krockel, and C. Hill.** 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. Int. J. Food Microbiol. **28:**169-185.

18. **Rodriguez, J. M., M. I. Martinez, N. Horn, and H. M. Dodd.** 2003. Heterologous production of bacteriocins by lactic acid bacteria. Int. J. Food Microbiol. **80:**101-106.

19. **Eijsink, V. G., M. Skeie, P. H. Middelhoven, M. B. Brurberg, and I. F. Nes.** 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. **64:**3275-3281.

20. **Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki.** 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. **24:**85-106.

21. **Héchard, Y., and H-G. Sahl.** 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie **84:**545-557.

22. **Moll, G. N., W. N. Konings, and A. J. M. Driessen.** 1999. Bacteriocins: mechanism of membrane insertion and pore formation. Antonie van Leeuwenhoek **76:**185-198.

23. **Moreno, M. R. F, P. Sarantinopoulos, E. Tsakalidou, and L. de Vuyst.** 2005. The role and application of enterococci in food and health. Int. J. Food Microbiol. **106(1):**1-24.

24. **Franz, C. M. A. P., and W. H. Holzapfel.** 2004. The genus *Enterococcus*: biotechnological and safety issues. In: Salminen, S., von Wright, A., A. Ouwenhand (Eds.), lactic acid bacteria: microbiological and functional aspects, third edition. Marcel Dekker Inc., New York, pp. 199-247.

25. **O'Mahony, A. T., F. O'Sullivan, Y. Walsh, A. Vaughan, M. Maher, G. F. Fitzerald, and D. van Sindersen.** 2000. Characterization and heterologous of antimicrobial producing lactic acid bacteria from malted barley. J. Inst. Brew. **106:**403-410.

26. **Giacometti, A., O. Cirioni, M. S. Del Prete, A. M. Paggi, M. M. D'Errico, and G. Scalise.** 2000. Combination studies between polycationic peptides and clinically used antibiotics against Gram-positive and Gram-negative bacteria. Peptides **21:**1155-1160.

27. **Ingham, A. B., M. Ford, R. J. Moore, and M. Tizard.** 2003. The bacteriocin piscicolin 126 retains antilisterial activity *in vivo*. J. Antimicrob. Chemother. **51:**1365-1371.

28. **Wachsman, M. B., M. E. Farias, E. Takeda, F. Sesma, A. P. De Ruiz Holgado, R. A. de Torres, and C. E. Coto.** 1999. Antiviral activity of enterocin CRL35 against herpes virus. Int. J. Antimicrob. Agents **12:**293-299.

29. **Wachsman, M. B., V. Castilla, A. P. De Ruiz Holgado, R. A. de Torres, F. Sesma and C. E. Coto.** 2003. Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication *in vitro*. Antiviral Res. **58:**17-24.

30. **Chen, H., and D. G. Hoover.** 2003. Bacteriocins and their food applications. Compr. Rev. Food Science Safety **2:**82-100.

31. **Chen, Y., R. D. Ludescher, and T. J. Montville.** 1997. Electrostatic interactions, but not the YGNGV consensus motif, govern the binding of pediocin PA-1 and its fragments to phospholipids vesicles. Appl. Environ. Microbiol. **63:**4770-4777.

32. **Guyonnet, D., C. Fremaux, Y. Catiempo, and J. M. Berjeaud.** 2000. Method for rapid purification of class IIa bacteriocins and comparison of their activities. Appl. Environ. Microbiol. **66:**1744-1748.

33. **Todorov, S. D., and L. M. T. Dicks.** 2005. Optimization of bacteriocin ST311LD production by *Enterococcus faecium* ST311LD, isolated from spoiled black olives. J. Microbiol. **43(4):**370-374.

34. **Parente, E., and A. Ricciardi.** 1994. Influence of pH on the production of enterocin 1146 during batch fermentation. Lett. Appl. Microbiol. **19:**12-15.

35. **Abriouel, H., E. Valdivia, A. Galvez, and M. Maqueda.** 2001. Influence of physico-chemical factors on the oligomerization and biological activity of bacteriocins AS-48. Curr. Microbiol. **42:**89-95.

36. **Leroy, F., M. R. Foulquie Moreno, and L. De Vuyst.** 2003. *Enterococcus faecium* RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation. Int. J. Food Microbiol. **88:**235-240.

37. **Herranz, C., J. M. Martinez, J. M. Rodriguez, P. E., and L. M. Cintas.** 2001. Optimization of enterocin P production by batch fermentation of *Enterococcus faecium* P13 at constant pH. Appl. Microbiol. Biotechnol. **56:**378-383.

38. **Parente, E., A. Ricciardi, and G. Addario.** 1994. Influence of pH on growth and bacteriocins production by *Lactococcus lactis* subsp. *lactis* 140VWC during batch fermentation. Appl. Microbiol. Biotechnol. **41:**388-394.

39. **De Vuyste, L., R. Callewaert, and K. Crabbe.** 1996. Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiol. **142:**817-827.

40. **Aasen, I. M., T. Moreto, T. Katla, L. Axelsson, and I. Storro.** 2000. Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. Appl. Microbiol. Biotechnol. **53:**159-166.

41. **Todorov, S., B. Gotcheva, X. Dousset, B. Onno, and I. Ivanova.** 2000. Influence of growth medium on bacteriocin production in *Lactobacillus plantarum* ST31. Biotechnol. Biotechnol. Eq. **14:**50-55.

42. **Audisto, M. C., G. Oliver, and M. C. Apella.** 2001. Effect of different complex carbon sources on growth and bacteriocin synthesis of *Enterococcus faecium*. Int. J. Food Microbiol. **63:**235-241.

43. **Sambrook, J. E., F. Eritsch, and J. Maniatis.** 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold spring Harbour, NY: Cold spring harbour Laboratory Press.

44. **Ivanova, I., V. Miteva, Ts. Stefanova, A. Pantev, I. Budakov, S. Danova, P. Moncheva, I. Nikolova, X. Dousset, and P. Boyaval.** 1998. Characterization of a bacteriocin produced by *Streptococcus thermophilus* 81. Int. J. Food. Microbiol. **42:**147-158.

45. **Todorov, S. D., and L. M. T. Dicks.** 2006. Parameters affecting the adsorption of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423 isolated from sorghum beer. J. Biotechnol. **1:**405-409.

46. **Todorov, D. T., M. B. Wachsman, H. Knoetze, M. Meincken, and L. M. T. Dicks.** 2005. An antibacterial and antiviral peptide produced by *Enterococcus mundtii* ST4V isolated from soya beans. Int. J. Antimicrob. Agents. **25:**508-513.

47. **De Man, J. C., M. Rogosa, and M. E. Sharp.** 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23:**130-135.

48. **Raibaud, P., M. Caulet, J. V. Galpin, and G. Mocquot.** 1961. Studies on the bacterial flora of the alimentary tract of pigs. II. Streptococci: selective enumeration and differentiation of the dominant group. J. Appl. Bacteriol. **24:**285-305.

49. **Ananou, S. and A. Ga´lvez, M. Martý´nez-Bueno, M. Maqueda and E. Valdivia.** 2005. Synergistic effect of enterocin AS-48 in combination with outer membrane permeabilizing treatments against *Escherichia coli* 0157:H7. J. Appl. Microbiol. **99:**1364-1372.

50. **Jennes, W., L. M. T. Dicks and D. J. Verwoerd.** 2000. Enterocin 012, a bacteriocin produced by *Enterococcus gallinarum* isolated from the intestinal tract of ostrich. J. Appl. Microbiol. **88:**349-357.

51. **Sparo, M. D., M. S. Castro, P. J. Andino, M. V. Lavigne, C. Ceriani, G. L. Gutie´ rrez, M. M. Ferna´ndez, M. C. De Marzi, E. L. Malchiodi and M. A. Manghi.** 2006. Partial characterization of enterocin MR99 from a corn silage isolate of *Enterococcus faecalis*. J. Appl. Microbiol. **100:**123-134.

52. **Todorov, S. D., and L. M. T. Dicks.** 2005. *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram-negative bacteria. Enzyme Microb. Technol. **36:**318-326.

53. **Cintas, L. M., P. Casaus, C. Herranz, L. S. Havaratein, H. Holo, P. E. Herna´ndez and I. F. Nes.** 2000. Biochemical and genetic evidence that *Enterococcus faecium* L50 produces enterocins L50A and L50B, the *sec*-dependent enterocin P. and a novel bacteriocin secreted without an N-terminal extension termed enterocin Q. J. Bacteriol. **182(23):**6806-6814.

54. **Yildrimim, Z., Y. K. Avsar, and M. Yildirim.** 2002. Factors affecting the adsorption of buchericin LB, a bacteriocin produced by *Lactobacillus* buchneri. Micrbiol. Res. **157:**103-107.

55. **Manca de Nadra, M. C., D. Sandino de Lamelas, and A. M. Strasser de Saad.** 1998. Pediocin N5p from *Pediococcus pentosaceus*: adsorption on bacterial strains. Int. J. Food Microbiol. **39:**79-85.

56. **Minahk, C. J., M. E. Farias, F. Sesma, and R. D. Morero.** 2000. Effect of enterocin CRL35 on *Listeria monocytogenes* cell membrane. FEMS Microbiol. Lett. **192:**79-83.

57. **Saavedra, L., C. Minahk, A. P. de Ruiz Holgado, and F. Sesma.** 2004. Enhancement of the enterocin CRL35 activity by synthetic peptide derived from the NH_2-terminal sequence. Antimicrob. Agents Chemother. **48:**2778-2781.

58. **Kawamoto, S., J. Shima, R. Sato, T. Educhi, S. Ohmomo, J. Shibato, N. Horikashi, K. Takeshita, and T. Sameshima.** 2002. Biochemical and genetic characterization of

mundticin KS, an antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. Appl. Environ. Microbiol. **68:**3830-3840.

59. **Bennik, M. H. J., B. Vanloo, R. Brasseur, L. G. M. Gorris, and E. J. Smid.** 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: Full characterization and interaction with target organism. Biochemical et biophysica Acta. **1373:**47-58.

60. **Herranz, C., L. M. Cintas, P. E. Hernandez, G. N. Moll, and J. M. Driessen.** 2001. Enterocin P causes potassium ion efflux from *Enterococcus faecium* T136 cells. Antimicrob. Agents Chemother. **45(3):**901-904.

61. **Herranz, C., Y. Chen, H-J. Chung, L. M. Cintas, P. E. Hernandez, T. J. Montville, and M. L. Chikindas.** 2001. Enterocin P selectively dissipates the membrane potential of *Enterococcus faecium* T136. Appl. Environ. Microbiol. **67(4):**1689-1692.

62. **Cintas, L. M., P. Casaus, L. S. Håvarstein, P.E. Hernández, and I.F. Nes.** 1997. Biochemical and genetic characterization of enterocin P, a novel *sec*-dependent bacteriocin from *Enterococcus Faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. **63:**4321-4330.

63. **Gálvez, A., E. Valdivia, H. Abriouel, E. Camafeita, E. M. M. Martínez-Bueno, and M. Maqueda.** 1998. Isolation and characterization of enterocin EJ97, a bacteriocin produced by *Enterococcus faecalis* EJ97. Arch. Microbiol. **171:**59-65.

64. **Verellen, T. L. J., G. Bruggeman, C. A. van Reenen, L. M. T. Dicks, and E. J. Vandamme.** 1998. Fermentation optimisation of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423. J. Ferment. Bioeng. **86:**174-179.

65. **Parente, E., C. Brienza, A. Ricciardi, and G. Addario.** 1997. Growth and bacteriocin production by *Enterococcus faecium* DPC1146 in batch and continuous culture. J. Ind. Microbiol. Biotechnol. **18:**62-67.

66. **Enan, G., A. A. Essawy, M. Uyttendaele, and J. Debevere.** 1996. Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization, production and bactericidal action of plantaricin UG1. Int. J. Microbiol. **30:**189-215.

67. **Moreno, M. R. F., M. C. Rea, T. M. Cogan, and L. De Vuyst.** 2003. Applicability of bacteriocin-producing *Enterococcus faecium* as a co-culture in Cheddar cheese manufacture. Int. J. Food Microbiol. **81:**73-84.

68. **Zendo, T., N. Eungruttanagorn, S. Fujioka, Y. Tashiro, K. Nomura, Y. Sera, G. Kobayashi, J. Nakayama, A. Ishizaki, and K. Sonomoto.** 2005. Identification and production of a bacteriocin from *Enterococcus mundtii* QU 2 isolated from soybean. J. Appl. Microbiol. **99:**1181-1190.

69. **Aymerich, T., M. G. Artigas, M. Garriga, J. M. Monfort, and M. Hugas.** 2000. Effect of sausage ingredients and additives on the production of enterocin A and B by *Enterococcus faecium* CTC492. Optimization of *in vitro* production and anti-listerial effect in dry fermented sausages. J. Appl. Microbiol. **88:**686-694.

70. **Keren, T., M. Yarmus, G. Halevy, and R. Shapira.** 2004. Immunodetection of the bacteriocin lacticin RM: analysis of the influence of temperature and Tween 80 on its expression and activity. Appl. Environ. Microbiol. **70:**2098-2104.

71. **Joosten, H. M. L. J., and M. Nun˜ ez.** 1995. Adsorption of nisin and enterocin 4 to polypropylene and glass surfaces and its prevention by Tween 80. Lett. Appl. Microbiol. **21:**389-392.

72. **Low, D. E.** 2001. Mechanisms of antimicrobial resistance. In: Niederman, M.S., Sarosi, G. A. and Glassroth, J. (Eds.). Respiratory Infections, 2nd ed. Lippincott Williams and Wilkens, Philadelphia. pp. 93-107.

TABLE 1. Antimicrobial activity of bacST4SA crude-extract

Pathogen	Temperature (°C)	Growth medium, incubation	Origin, collection	Antimicrobial activity
Acinetobacter baumannii				
16	37	BHI, aerobic	Human middle ear	+
19	37	BHI, aerobic	Human middle ear	-
Enterococcus faecalis				
20	37	BHI, aerobic	Human middle ear	+++
21	37	BHI, aerobic	Human middle ear	+
Enterococcus faecium HKLHS	30	MRS, aerobic		++++
Haemophilus influenza				
C	37	CBA, aerobic	Human middle ear	-
Pseudomonas aeruginosa				
8, 14, 25	37	BHI, aerobic	Human middle ear	-
G, BG	37	BHI, aerobic	Human middle ear	(+)
I, J	37	Bld, aerobic	Human middle ear	(+++)
B	37	BHI, aerobic	Nasal cavity	(+++)
E	37	BHI, aerobic	Nasal cavity	(+)
Staphylococcus aureus				
13	37	BHI, aerobic	Human middle ear	-
36	37	BHI, aerobic	Human middle ear	+++
Staphylococcus carnosus				
LMG 13567	37	BHI, aerobic	Human middle ear	-
Streptococcus pneumoniae				
A, D, 10	37	BHI/Bld, aerobic	Human middle ear	-
29		BHI, aerobic	Human middle ear	+++
27, 40		BHI, aerobic	Human middle ear	++++
Unidentified Gram-positive				
A, BW, DW, F, G, H		BHI, aerobic	Human middle ear	++++

- = no zones, + = diameter between 1 and 11mm, ++ = diameter between 12 and 16mm, +++ = diameter between 17 and 21mm, ++++ = diameter of at least 22mm. LMG = Laboratory of Microbiology, University of Gent, Belgium. Bld = blood agar, CBA = chocolate blood agar. Results in parenthesis refer to activity with some resistance.

TABLE 2. Effect of bacST4SA cell-free supernatant on cell permeability

Pathogens	Cellular leakage (OD_{260})	β-galactosidase-leakage (OD_{420})
Streptococcus pneumoniae		
27	1.670	No leakage
29	1.182	No leakage
40	0.801	No leakage
Enterococcus faecalis		
20	0.930	No leakage
Staphylococcus aureus		
36	0.892	No leakage
Gram-positive middle ear isolates (unidentified)		
BW	1.186	0.370
DW	1.153	0.293
F	1.428	0.318
H	1.559	0.235
G	0.751	0.373
Enterococcus faecium HKLHS	1.395	No leakage

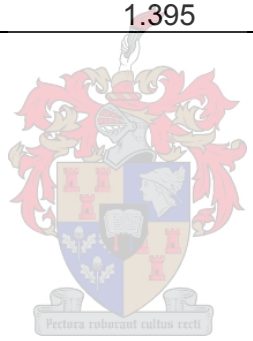


TABLE 3. Effect of MRS components and vitamins on bacST4SA production.

Component	Concentration (g/l)	Bacteriocin activity (AU/ml)	Bacteriocin activity* (%)
Carbon Source			
Glucose (control)	20.0	51 200	100
Saccharose	20.0	51 200	100
Lactose	20.0	25 600	50
Maltose	20.0	51 200	100
Mannose	20.0	25 600	50
Nitrogen source			
Tryptone	20.0	102 400	200
Meat extract	20.0	51 200	100
Yeast extract	20.0	102 400	200
Tryptone	0.0	800	2.0
Tryptone	0.5	3200	6.25
Tryptone	1.0	6400	12.5
Tryptone	2.0, 3.0	12 800	25
Tryptone	4.0, 5.0, 10.0, 30.0, 40.0	25 600	50
Tryptone	15.0	51 200	100
Meat and yeast extract	10.0 + 10.0	51 200	100
Tryptone and meat extract	12.5 + 7.5	51 200	100
Tryptone and yeast extract	12.5 + 7.5	102 400	200
Mineral salts			
tri-Ammonium Citrate	0.0, 0.5, 1.0, 5.0	12 800	25
	2.0	51 200	100
	10.0	25 600	50
Magnesium sulphate	0.0	6400	12.5
	0.1	51 200	100
	0.2	12 800	25
	0.5	3200	6.25
	1.0	800	2.0
Manganese sulphate	0.0, 0.1	12 800	25
	0.05	51 200	100
	0.2, 0.5	6400	12.5
Sodium acetate	0.0, 1.0	6400	12.5
	2.0	12 800	25
	5.0	51 200	100

TABLE 3. (Continued)

Component	Concentration (g/l)	Bacteriocin activity (AU/ml)	Bacteriocin activity* (%)
	10.0	25 600	50
K_2HPO_4	2.0, 5.0	51 200	100
	10.0, 20.0	102 400	200
	50.0	6400	12.5
KH_2PO_4	2.0	25 600	50
Glycerol	0.0, 1.0	51 200	100
	2.0, 5.0, 10.0	25 600	50
	20.0, 50.0	12 800	25
Vitamins			
Cyanocobalamin	0.001	51 200	100
DL-6,8-thioctic acid	0.001	102 400	200
L-ascorbic acid	0.001	102 400	200
Phylloquinone	0.001	25 600	50
Thiamine	0.001	102 400	200
MRS broth	(control)	51 200	100

*Compared to the highest activity (AU/ml), as recorded with the control (MRS, Biolab)

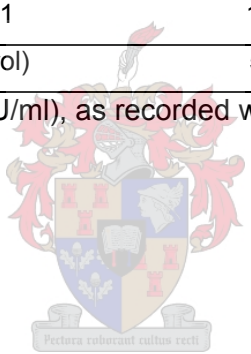


TABLE 4. Comparison of bacST4SA crude extract with other alternative treatments

Treatments	Inhibition of bacterial growth		
	E. faecium HKLHS	*P. aeruginosa* J	*S. pneumoniae* 27
Eye/Otic drops			
CiproBay®HC Otic [1]	++	+++	++
Exocin (Oflaxacin, 0.3%) [2]	++	+++	+++
Sofradex® [3]	+	++	-
Tobrex® (Tobramycin) [1]	+	+++	-
Nasal Spray			
Flomist [4], Vibrocil [5]	+	+	+
Antibiotics [6]			
Amikacin (30 µg)	-	+++	-
Amoxicillin (10 µg)	+	++	+++
Augmentin (30 µg)	+	(+++)	+++
Cefepime (30 µg)	-	+++	+
Cefotaxime (30 µg), Ceftriaxone (30 µg), Furazolidone (50 µg), Rifampicin (5 µg)	+	+	+
Ceftazidime (30 µg), Streptomycin (25 µg), Tobramycin (10 µg),	-	++	-
Cefuroxime (30 µg), Metronidazole (5 µg), Oxacillin (1 µg), Sulfafurazole (25 µg), Sulfamethoxazole (25 µg), Trimethoprim (25 µg)	-	-	-
Chloramphenicol (10 µg)	++	(++)	+
Ciprofloxacin (5 µg)	+	(++++)	+
Clindamycin (2 µg)	+	-	-
Compound sulfonamides (300 µg)	-	(+++)	-
Erythromycin (5 µg)	+	+	++
Fusidic acid (10 µg)	+	-	++
Nalidixic acid (30 µg)	-	+	(+)
Nitrofurantoin (300 µg)	+	+++	++
Ofloxacin (5 µg)	+	++	+
Tetracycline (30 µg)	+++	(++)	+++
Vancomycin (30 µg)	+	-	+
Bacteriocin crude extract			
BacST4SA (1 638 400 AU/ml)	+++	(+++)	+++

- = no zones, + = diameter 1 - 11mm, ++ = diameter 12 - 16mm, +++ = diameter 17 - 21mm, ++++ = diameter at least 22mm. Results in brackets refer to resistance with little growth. Antimicrobial agents were supplied by Alcon Laboratories, South Africa (1), Allergan Pharmaceuticals South Africa (2), Aventis Pharma, South Africa (3), Cipla Medpro, South Africa (4), Novartis SA, South Africa (5) and Oxoid, New Hampshire, England (6).

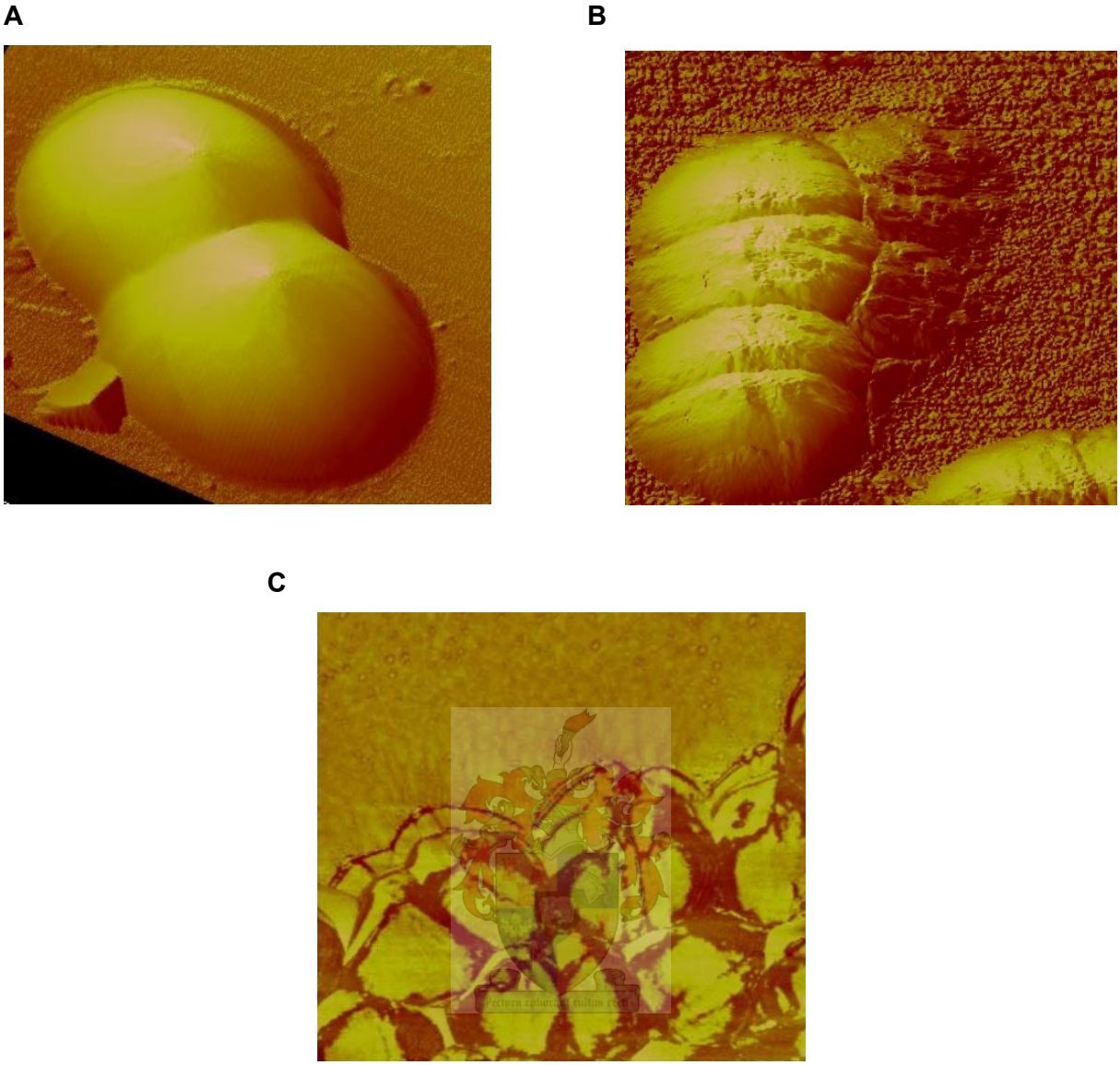


Fig. 1. Atomic force microscopy images of *E. faecium* HKLHS before (A) and after (B and C) treatment with bacST4SA (49 152 AU/ml). Leakage of cell contents from damaged cells is clearly visible (C).

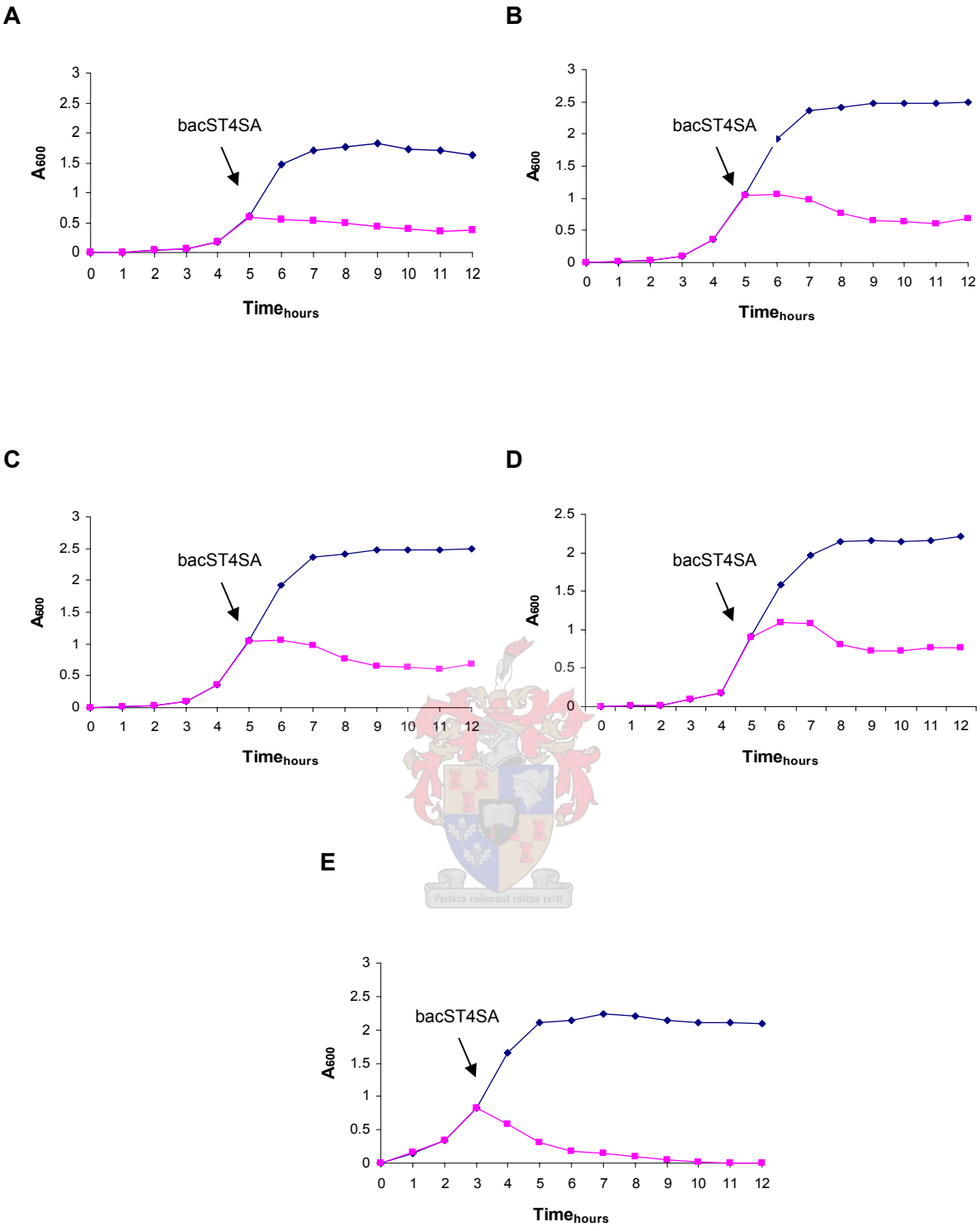


Fig. 2. Effect of bacST4SA crude-extract (1 638 400 AU/ml) on (A) *S. pneumoniae* 40, (B) middle ear isolate F, (C) middle ear isolate BW (D) middle ear isolate H and (E) *E. faecium* HKLHS. Symbols: ◆ = growth in the absence of bacST4SA, ■ = growth in the presence of bacST4SA.

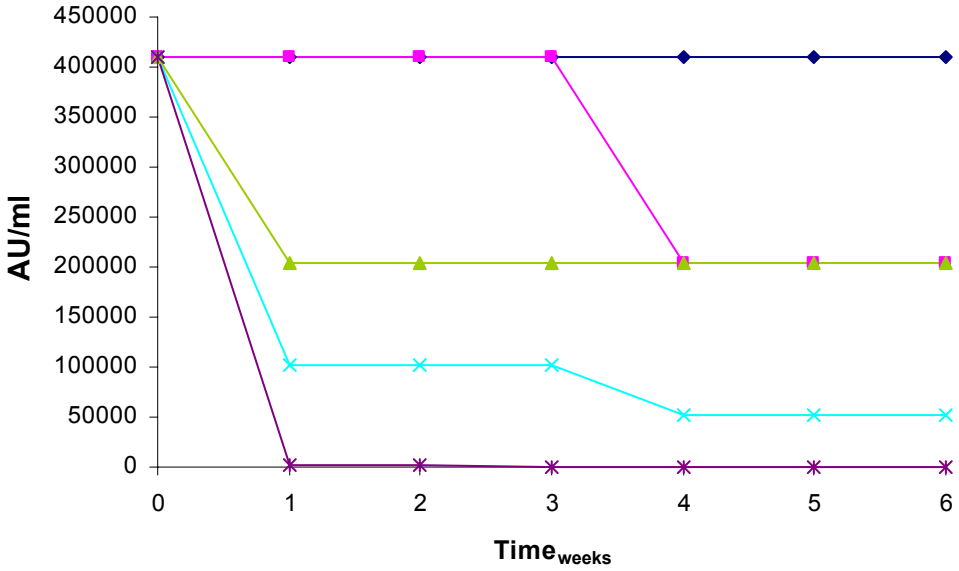


Fig. 3. Effect of temperature on the stability of bacST4SA. Symbols: ◆ = -20°C, ■ = 4°C, ▲ = 25°C, x = 37°C, ✳ = 60°C.

DISCUSSION AND CONCLUSIONS

Acute otitis media is a common infectious disease of infants and children, and treatment with antimicrobial agents is familiar (Segal *et al.,* 2005). After treatment, a risk of selection of antibiotic-resistant bacteria in the oropharynx and serious alternations of the ecological balance of the normal microflora is possible, opening possibilities for the re-establishment of pathogens in the middle ear (Lisby-Sutch *et al*., 1990; Brook and Foote, 1997; Faden *et al.,* 1997; Brook and Gober, 1998; Klein, 1999; Block *et al.,* 2001; Faden, 2001; Rovers *et al.,* 2004; Brook and Gober, 2005). An extensive focus is the bacterial interference (BI) of natural nasopharyngeal flora with their ability to produce antibacterial substances inhibiting pathogens (Brook, 2005). Important bacteriocin-producers are alpha-hemolytic streptococci, mostly *Streptococcus mitis* and *Streptococcus sanguis,* anaerobic streptococci (*Peptostreptococcus anaerobius* and *Prevotella melaninogenica*) and non-haemolytic streptococci (Sanders *et al.,* 1977; Berstein *et al.,* 1993; Bernstein *et al.,* 1994; Brook, 2005). A bacteriocin-like substance produced by *Streptococcus salivarius,* a non-pathogenic strain isolated from the oral cavity of humans, inhibited the growth of *Streptococcus pyogenes*, and revealed *in vitro* inhibitory activity against Gram-negative pathogens such as *Moraxella catarrhalis* and *Haemophilus influenzae* (Dierksen and Tagg, 2000; Walls *et al.,* 2003).

In this study, a lactic acid bacterium was isolated from soya beans and identified as an *Enterococcus mundtii* ST4SA. A heat stable, cationic and hydrophobic bacteriocin, bacST4SA, was produced, identified and characterized. The bacST4SA gene cluster is located on a 50-kb plasmid and consists of three open reading frames (ORFs). The first ORF (structural gene) encodes a 58-amino acid peptide that consists of a 15-amino acid leader- and a 43-amino acid mature peptide. The structural gene is homologous to mundticin AT06, mundticin KS and bacteriocin QU 2, produced by *E. mundtii* AT06 (Bennik *et al.,* 1998), *E. mundtii* NFRI 7393 (Kawamoto *et al.,* 2002) and *E. mundtii* QU 2 (Zendo *et al.,* 2005), respectively. The leader peptide of bacST4SA differ from that of enterocin CRL35 of *E. mundtii* CRL 35 by two amino acids (Farias *et al.,* 1996; Saavedra *et al.,* 2004). The mature peptide is typical of all class IIa bacteriocins, by the presence of a conserved YGNGV consensus motif, a disulphide bridge linking cys^{9} and cys^{14}, and a high number of non-polar amino acids (Nes *et al.,* 1996; Eijsink *et al.,* 1998; Ennahar *et al.,* 2000). The molecular weight of the mature peptide was determined at 3950 Da. The second ORF encodes a 674-amino acid ATP-dependent transporter protein and is located downstream from the structural gene. The ATP-dependent transporter consisted of a peptidase C39B-, an ABC-membrane

transporter- (revealing five primary transmembrane helixes and one secondary transmembrane helix) and an ABC-DLP family domain. The second ORF is 98.9% homologous to mundticin KS (Kawamoto *et al.,* 2002) and 99.25% to enterocin CRL35 (Saavedra *et al.,* 2004). The third ORF encodes a 98-amino acid immunity protein and is located immediately downstream of the ATP-dependent transporter gene. The immunity gene is completely homologous to enterocin CRL35 (Saavedra *et al.,* 2004) and 96.9% homologous to mundticin KS (Kawamoto *et al.,* 2002).

The proteinaceous nature of bacST4SA was confirmed by it's sensitivity to proteolytic enzymes. BacST4SA is stable in acidic conditions (pH 2.0), at high temperatures (121 °C for 15 min), and in the presence of middle ear fluid and blood. Optimal bacST4SA production (51 200 AU/ml) was recorded after 14 h of growth in MRS broth, pH 6.5, and inhibits the growth of *Bacillus cereus, Clostridium tyrobutyricum, Enterococcus faecalis, Enterococcus faecium, Lactobacillus sakei, Pediococcus* sp., *Propionibacterium* spp., *Streptococcus caprinus* and the food-borne pathogen *Listeria monocytogenes*. A two-fold increase in bacST4SA was observed in MRS broth supplemented with tryptone, yeast extract, or a combination of tryptone and yeast extract, K_2HPO_4 (10.0 or 20.0 g/l), DL-6,8-thoictic acid, L-ascorbic acid, and thiamine, respectively. A 64-fold increase in activity (1 638 400 AU/ml) was revealed after 80% saturated ammonium sulphate precipitation, followed by dialysis and lyophilisation, and displayed antimicrobial activity against strains of middle ear isolated pathogens *Acitenobacter baumannii*, *E. faecalis*, *E. faecium*, *S. aureus*, *S. pneumoniae*, and unidentified middle ear isolates A, BW, DW, F, G, and H. BacST4SA inhibits the growth Gram-negative strains of *P. aeruginosa*, although some resistance was observed. In general, the inability of bacST4SA to inhibit Gram-negative bacteria may be due to the protective outer membrane of Gram-negative strains. The outer membrane excludes hydrophobic macromolecules and substances from entering Gram-negative cells, preventing bacST4SA to reach the target (Nikaido, 1996). Future research will be to overcome this penetration barrier, rendering Gram-negative pathogens, currently resistant to bacST4SA, sensitive to bacST4SA. Sensitization can be brought about by metal-chelating organic acids, such as EDTA, removing stabilizing cations from the outer membrane, resulting in the loss of LPS, decreasing the protective function as penetration barrier (Stevens *et al.,* 1991; Delves-Broughton, 1993; Schved *et al.,* 1994; Cutter and Siragusa, 1995; Boziaris and Adams, 1999; Helander and Mattila-Sandholm, 2000).

The mode of activity of bacST4SA was bactericidal towards *E. faecium* HKLHS and bacteriostatic towards *S. pneumoniae* 40, middle ear isolate F, BW and H. The primary site

of action is the cytoplasmic membrane, resulting in the formation of pores, dissipation of the proton motive force (PMF) and leakage of intracellular molecules and substances. Interactions with the cytoplasmic membrane are by adsorption of bacST4SA to specific receptors of sensitive target strains (Ananou *et al.,* 2005; Zendo *et al.,* 2005). The percentage adsorption of bacST4SA might depend on the amount and type of receptors available on the cytoplasmic membrane for adhesion, and may be influenced by several environmental factors (Ananou *et al.,* 2005; Zendo *et al.,* 2005). The effect of environmental factors on bacST4SA, such as pH and temperature, will have to be studied. In general, maximum adsorption occurs at neutral pH, with a decrease in adsorption as the culture pH decreases (Yang *et al.,* 1992). Low pH increases bacteriocin activity by changing the oligomerization of the bacteriocin and the electric charge on the surface of the target organism (Abriouel *et al.,* 2001a; Abriouel *et al.,* 2001b; Ananou *et al.,* 2005). The increase in activity may be due to an increase in the net charge of the bacteriocin molecule, facilitating interaction with the negatively charges of the cytoplasmic membrane of the bacteria and translocation through the cell wall (Abriouel *et al.,* 2001a; Abriouel *et al.,* 2001b). The effect bacST4SA has on the dissipation of the pH gradient and transmembrane potential, thus PMF dissipation, will have to be studied. Target-cell binding, potency and specificity of bacST4SA may also be investigated using site-directed *in vitro* mutagenesis (Kazazic *et al.,* 2002).

BacST4SA revealed similar to higher activity towards pathogens, when compared with other antimicrobial alternatives. The application of bacST4SA in medicine is promising, since encouraging examples of other cationic peptides have been reported (Giacometti *et al.,* 1999; Giacometti *et al.,* 2000 ; Brumfitt *et al.,* 2002 ; Ingham *et al.,* 2003) and bacST4SA retained activity in the presence of middle ear fluid and blood. However, for bacST4SA to be used as a new alternative for the treatment of otitis media, clinical research needs to be conducted. Strategies to enhance the activity of bacST4SA will be considered and can be approached in three different ways. The first approach is the identification and development of short peptides that exert strong enhancement on the antimicrobial activity of bacST4SA, and the design of variants with improved characteristics. The second approach is the genetic engineering of bacST4SA, by cloning bacST4SA and other antimicrobial or antifungal peptides into a suitable expression vector, enhancing its activity against pathogens of the middle ear. Thirdly, the synergistic effect of antibiotics and sub-lethal concentrations of bacST4SA to enhance activity against pathogens and the role played by proton gradient extrusion pumps should be investigated.

Studies on pharmacological profiles, immunogenic and toxicity, and route of administration are important if bacST4SA is to be used in clinical medicine. Pharmacological profiles are divided into two components: 1) pharmacokinetics, including the dosing regimen, adsorption of bacST4SA to middle ear mucosa, and its distribution and elimination, which determine the time course of bacST4SA concentrations in serum, middle ear fluid, and in the mucosal tissues that surround the middle ear fluid; and 2) pharmacodynamics, involving the association between concentrations of bacST4SA at the site of infection and it's antimicrobial effect (Dagan and Leibovitz; 2002). *In vivo* studies are needed to conclude whether bacST4SA remains active against middle ear pathogens. The effect of bacST4SA on the ecological balance of normal oropharyngeal microflora should be studied.

REFERENCES

Abriouel, H., J. Sanchez-Gonzalez, M. Maqueda, A. Gálvez, E. Valdivia, and M.J. Gálvez-Ruiz. 2001a. Monolayers characteristics of bacteriocin AS-48, pH effect and interactions with dipalmitoyl phosphatidic acid at the air-water interface. J. Colloid Interface Sci. 233, 306-312.

Abriouel, H., E. Valdivia, A. Gálvez, and M. Maqueda. 2001b. Influence of physico-chemical factors on the oligomerization and biological activity of bacteriocin AS-48. Curr. Microbiol. 42, 89-95.

Ananou, S., A. Gálvez, M. Martynez-Bueno, M. Maqueda, and E. Valdivia. 2005. Synergistic effect of enterocin AS-48 in combination with outer membrane permeabilizing treatments against *Escherichia coli* 0157:H7. J. Appl. Microbiol. 99, 1364-1372.

Bennik, M.H.J., B. Vanloo, R. Brasseur, L.G.M. Gorris, and E.J. Smid. 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: Full characterization and interaction with target organism. Biochem. Biophys. Acta 1373, 47-58.

Bernstein, J.M., H.F. Faden, D.M. Dryja, and J. Wactawski-Wende. 1993. Micro-ecology of the nasopharyngeal bacterial flora in otitis-prone and non-otitis-prone children. Acta Otolaryngol. 113, 88-92.

Bernstein, J.M., S. Sagahtaheri-Altaie, D.M. Dryjd, and J. Wactawski-Wende. 1994. Bacterial interference in nasopharyngeal bacterial flora of otitis-prone and non-otitis-prone children. Acta oto-laryngologica, Belgium 48, 1-9.

Block, S.L., C.L. Harrison, J. Hedrick, R. Tyler, A. Smith, and R. Hedrick. 2001. Restricted use of antibiotic prophylaxis for recurrent acute otitis media in the era of penicillin non-susceptible *Streptococcus pneumoniae*. Int. J. Pediatr. Otorhinolaryngol. 61(1), 47-60.

Boziaris, I.S., and M.R. Adams. 1999. Effect of chelators and nisin produced *in situ* on inhibition and inactivation of Gram negatives. J. Food Microbiol. 53, 105-113.

Brook, I. 2005. The role of bacterial interference in otitis, sinusitis, and tonsillitis. Otolaryngol. Head Neck Surg. 133, 139-146.

Brook, I., and P.A. Foote. 1997. Bacterial interference and beta-lactamase-producing bacteria in the adenoids after antimicrobial therapy. Rev. Infect. Dis. 25, 493.

Brook, I, and A.E. Gober. 1998. Bacterial interference in the nasopharynx following antimicrobial therapy of acute otitis media. J. Antimicrob. Chemoth. 41, 489-492.

Brook, I., and A.E. Gober. 2005. Antimicrobial resistance in the nasopharyngeal flora of children with acute otitis media and otitis media recurring after amoxicillin therapy. J. Med. Microbiol. 54, 83-85.

Brumfitt, W., M. R. J. Salton, and J. M. T. Hamilton-Miller. 2002. Nisin, alone and combined with peptidoglycan-moduling antibiotics: activity against methillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. J. Antimicrob. Chemother. 50, 731-734.

Cutter, C.N., and G.R. Siragusa. 1995. Population reductions of Gram-negative pathogens following treatment with nisin and chelators under various conditions. J. Food Prot. 58, 977-983.

Dagan, R., and E. Leibovitz. 2002. Bacterial eradication in the treatment of otitis media. Lancet Infect. Dis. 2, 593-604.

Delves-Broughton, J. 1993. The use of EDTA to enhance the efficacy of nisin towards Gram-negative bacteria. Int. Biodeterior. Biodegr. 32, 87-97.

Dierksen, K. and J. Tagg. 2000. The influence of indigenous bacteriocin-producing *Streptococcus salivarius* on the acquisition of *Streptococcus pyogenes* by primary school children in Dunedin, New Zealand. In: Martin, D. and Tagg, J. (Eds.). Streptococci and Streptococcal Disease entering the New Millennium. Securacopy, Wellington, New Zealand. pp. 81-85.

Eijsink, V.G., M. Skeie, P.H. Middelhoven, M.B. Brurberg, and I.F. Nes. 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. 64, 3275-3281.

Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. 24, 85-106.

Faden, H. 2001. The microbiologic and immunologic basis for recurrent otitis media in children. Eur. J. Pediatr. 160(7), 407-413

Faden, H., L. Duffy, and R. Wasielewski. 1997. Relationship between nasopharyngeal colonization and the development of otitis media in children. J. Infect. Dis. 175, 1440-1445.

Farias, M.E., R.N. Farias, A.P. de Ruiz Holgado, and F. Sesma. 1996. Purification and N-terminal amino acid sequence of enterocin CRL 35, a 'pediocin-like' bacteriocin produced by *Enterococcus faecium* CRL 35. Lett. Appl. Microbiol. 22, 417-419.

Giacometti, A., O. Cirioni, F. Barchiesi, M. Fortuna, and G. Scalise. 1999. In-vitro activity of cationic peptides alone and in combination with clinical used antimicrobial agents against *Pseudomonas aeruginosa.* J. Antimicrob. Chemother. 44, 641-645.

Giacometti, A., O. Cirioni, M.S. Del Prete, A.M. Paggi, M.M. D'Errico, and G. Scalise. 2000. Combination studies between polycationic peptides and clinically used antibiotics against Gram-positive and Gram-negative bacteria. Peptides 21, 1155-1160.

Helander, I.M., and T. Mattila-Sandholm. 2000. Permeability of the Gram-negative bacterial outer membrane with special reference to nisin. Int. J. Food Microbiol. 60, 153-161.

Ingham, A.B., M. Ford, R.J. Moore, and M. Tizard. 2003. The bacteriocin piscicolin 126 retains antilisterial activity *in vivo*. J. Antimicrob. Chemother. 51, 1365-1371.

Kawamoto, S., J. Shima, R. Sato, T. Educhi, S. Ohmomo, J. Shibato, N. Horikashi, K. Takeshita, and T. Sameshima. 2002. Biochemical and genetic characterization of mundticin KS, an antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. Appl. Environ. Microbiol. 68, 3830-3840.

Kazazic, M., J. Nissen-Meyer, and G. Fimland. 2002. Mutational analysis of the role of charged residues in target-cell binding, potency and specificity of pediocin-like bacteriocin sakacin P. Microbiol. 148, 2019-2027.

Klein, J.O. 1999. Management of acute otitis media in an era of increasing antibiotic resistance. Int. J. Pediatr. Otorhinolaryngol. 49(1), S15-S17.

Lisby-Sutch, S., M.A. Nemec-Dwyer, R.G. Deeter, and S.M. Gaur. 1990. Therapy of otitis media. Clin. Pharmacol. 9, 15-34.

Nes, I.F., B.D. Diep, L.S. Havarstein, M.B. Brurberg, V. Eijsink, and H. Holo. 1996. Biosynthesis of bacteriocins of lactic acid bacteria. Antonie van Leeuwenhoek 70, 113-128.

Nikaido, H. 1996. Outermembrane. In: Neidhardt, F. C (Ed.), *Escherichia coli* and *Salmonella*. Cellular and molecular biology. ASM Press, Washington, DC, pp. 29-47.

Rovers, M.M., A.G. Schilder, G.A. Zielhuis, and R.M. Rosenfeld. 2004. Otitis media. Lancet 363(9407), 465-473.

Saavedra, L., C. Minahk, A.P. de Ruiz Holgado, and F. Sesma. 2004. Enhancement of the enterocin CRL35 activity by synthetic peptide derived from the NH_2-terminal sequence. Antimicrob. Agents Chemother. 48, 2778-2781.

Sanders, C.C., G.E. Nelson, and W.E. Sanders. 1977. Bacterial interference. IV. Epidemiologic determinants of the antagonistic activity of the normal throat flora against Group A streptococci. Infect. Immun. 16, 599-603.

Schved, F., Y. Henis, and B.J. Juven. 1994. Response of spheroplasts and chelator-permeabilized cells of Gram-negative bacteria to the action of bacteriocins pediocin SJ-1 and nisin. Int. J. Food Microbiol. 21, 305-314.

Segal, N., E. Leibovitz, R. Dagan, and A. Lieberman. 2005. Acute otitis media diagnosis and treatment in the era of antibiotic resistant bacteria: updated clinical practice guidelines. Int. J. Otorhinolaryngol. 69, 1311-1319.

Stevens, K.A., B.W. Sheldon, N.A. Klapes, and T.R. Klaenhammer. 1991. Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. Appl. Environ. Microbiol. 57, 3613-3615.

Walls, T., D. Power, and J. Tagg. 2003. Bacteriocin-like substance (BLIS) production by the normal flora of the nasopharynx: potential to protect against otitis media? J. Med. Microbiol. 52, 829-833.

Yang, R., M. Johnson, and B. Ray. 1992. A novel method to extract large amounts of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. 58, 3355-3359.

Zendo, T., N. Eungruttanagorn, S. Fujioka, Y. Tashiro, K. Nomura, Y. Sera, G. Kobayashi, J. Nakayama, A. Ishizaki, and K. Sonomoto. 2005. Identification and production of a bacteriocin from *Enterococcus mundtii* QU 2 isolated from soybean. J. Appl. Microbiol. 99, 1181-1190.