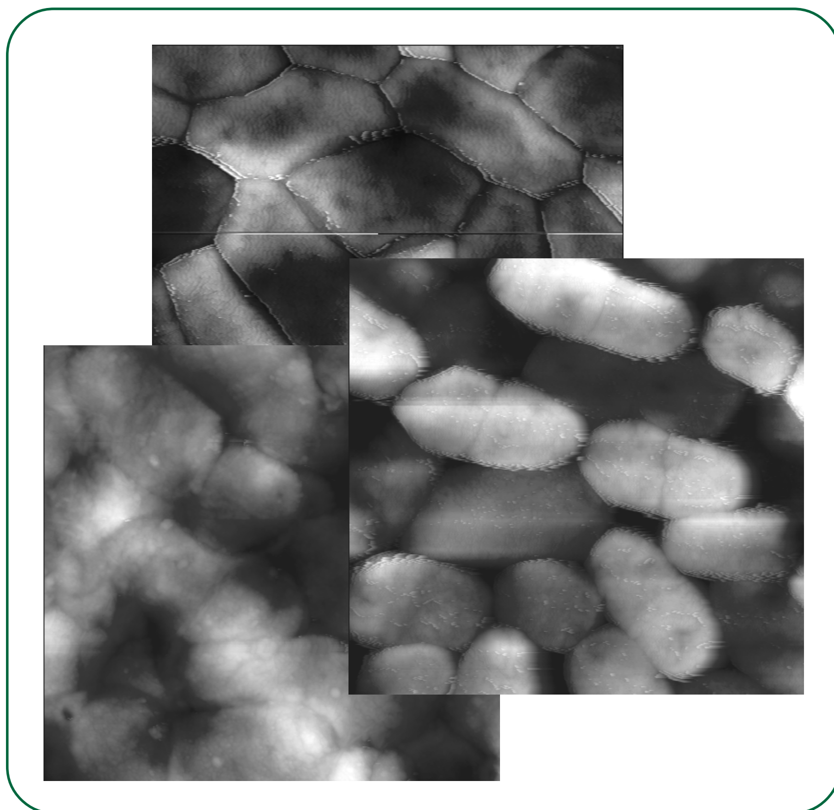
ESPOO 2007 VTT PUBLICATIONS 638



Hanna-Leena Alakomi

Weakening of the Gram-negative bacterial outer membrane

A tool for increasing microbiological safety

VTT

VTT PUBLICATIONS 638

Weakening of the Gram-negative bacterial outer membrane

A tool for increasing microbiological safety

Hanna-Leena Alakomi

Division of Microbiology,
Department of Applied Chemistry and Microbiology
University of Helsinki

Academic Dissertation in Microbiology

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in Auditorium 2 at Viikki Campus, Info center, Viikinkaari 11, on June 1st, 2007, at 12 o'clock noon.

ISBN 978-951-38-7014-0 (soft back ed.)
ISSN 1235-0621 (soft back ed.)

ISBN 978-951-38-7015-7 (URL: http://www.vtt.fi/publications/index.jsp)
ISSN 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)

Copyright © VTT Technical Research Centre of Finland 2007

JULKAISIJA – UTGIVARE – PUBLISHER

VTT, Vuorimiehentie 3, PL 1000, 02044 VTT
puh. vaihde 020 722 111, faksi 020 722 4374

VTT, Bergsmansvägen 3, PB 1000, 02044 VTT
tel. växel 020 722 111, fax 020 722 4374

VTT Technical Research Centre of Finland, Vuorimiehentie 3, P.O.Box 1000, FI-02044 VTT, Finland
phone internat. +358 20 722 111, fax + 358 20 722 4374

VTT, Tietotie 2, PL 1000, 02044 VTT
puh. vaihde 020 722 111, faksi 020 722 7071

VTT, Datavägen 2, PB 1000, 02044 VTT
tel. växel 020 722 111, fax 020 722 7071

VTT Technical Research Centre of Finland, Tietotie 2, P.O. Box 1000, FI-02044 VTT, Finland
phone internat. +358 20 722 111, fax +358 20 722 7071

Front cover: Atomic force microscopy images of *Pseudomonas* sp. E-02106 after control (back image), ethylenediaminetetraacetic acid (EDTA, front image) and polyethylenimine (PEI, middle image) treatments. Topographic images of the control *Pseudomonas* sp. E-012106 cells revealed a compact and smooth surface without notable ruptures or pores on the cell surface. The surfaces of the 1 mM EDTA-treated cells visualized in topographic images were rough and the outer membrane surface appeared damaged, indicating release of LPS and weakening of OM structure. Treatment of the cells with PEI flocculated the *Pseudomonas* cells, causing aggregation and adhesion. In addition, cells were swollen, with increased cell surface area and visible bulges (Paper IV).

Technical editing Anni Kääriäinen

Edita Prima Oy, Helsinki 2007

Supervisors: Docent Ilkka Helander
Department of Applied Chemistry and Microbiology
University of Helsinki
Helsinki, Finland

Docent Maria Saarela
VTT Technical Research Centre of Finland
Espoo, Finland

Reviewers: Professor Mikael Skurnik
University of Helsinki
Haartman Institute
Helsinki, Finland

Professor Airi Palva
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Opponent: Professor Dr. Otto Holst
Division of Structural Biochemistry
Forschungszentrum Borstel
Leibnitz-Center for Medicine and Biosciences
Borstel, Germany

Alakomi, Hanna-Leena. Weakening of the Gram-negative bacterial outer membrane. A tool for increasing microbiological safety [Gram-negatiivisten bakteerien ulkokalvon heikentäminen. Keino parantaa mikrobiologista turvallisuutta]. Espoo 2007. VTT Publications 638. 95 p. + app. 37 p.

Keywords Gram-negative bacteria, bacterial membrane, outer membranes, permeabilization, chelators, destabilizers, organic acids, EDTA, ethylenediaminetetraacetic acid, polyethylenimine

Abstract

Gram-negative bacteria are harmful in various surroundings. In the food industy their metabolites are a potential cause of spoilage and this group also includes many severe or potential pathogens. Due to their ability to produce biofilms Gram-negative bacteria also cause problems in many industrial processes as well as in clinical surroundings. Control of Gram-negative bacteria is hampered by the outer membrane (OM) in the outermost layer of the cells. This layer is an intrinsic barrier for many hydrophobic agents and macromolecules. Permeabilizers are compounds that weaken the OM and can thus increase the activity of antimicrobials by facilitating entry into the cells of external substances capable of inhibiting or destroying cellular funcions.

The work described in this thesis demonstrates that lactic acid acts as a permeabilizer and destabilizes the OM of Gram-negative bacteria. In addition, organic acids present in berries, i.e. malic, sorbic and benzoic acids, were shown to weaken the OM of Gram-negative bacteria. Microbial colonic degradation products of plant-derived phenolic compounds (e.g. 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid and 3-hydroxyphenylpropionic acid) efficiently destabilized OM of *Salmonella.* The studies increase our understanding of the mechanism of action of the classical chelator, ethylenediaminetetraacetic acid (EDTA). In addition, the results indicate that the biocidic activity of benzalkonium chloride against *Pseudomonas* can be increased by combined use with polyethylenimine (PEI). In addition to PEI, several other potential permeabilizers, such as succimer, were shown to destabilize the OM of Gram-negative bacteria. Furthermore, combination of the results obtained from various permeability assays (e.g. uptake of a hydrophobic probe, sensitization to hydrophobic antibiotics and detergents, release of lipopolysaccharide (LPS) and LPS-specific fatty acids) and atomic force microscopy (AFM) image results increases our knowledge of the action of permeabilizers.

Alakomi, Hanna-Leena. Weakening of the Gram-negative bacterial outer membrane. A tool for increasing microbiological safety [Gram-negatiivisten bakteerien ulkokalvon heikentäminen. Keino parantaa mikrobiologista turvallisuutta]. Espoo 2007. VTT Publications 638. 95 s. + liitt. 37 s.

Avainsanat Gram-negative bacteria, bacterial membranes, outer membrane, permeabilization, chelators, destabilizers, organic acids, EDTA, ethylenediaminetetraacetic acid, polyethylenimine

Tiivistelmä

Gram-negatiivisten bakteerien ryhmään kuuluu laaja joukko haittamikrobeja, esimerkiksi potentiaalisia patogeeneja (kuten salmonella) ja elintarvikkeissa esiintyviä pilaajamikrobeja. Monille Gram-negatiivisille bakteereille on ominaista kyky kasvaa pinnoilla ja muodostaa biofilmiä, minkä vuoksi ne aiheuttavat ongelmia prosessiteollisuudessa sekä kliinisissä ympäristöissä. Gram-negatiivisille bakteereille ominainen ulkokalvorakenne heikentää monien antimikrobisten yhdisteiden kulkeutumista solun sisään, minkä vuoksi Gram-negatiivisten bakteereiden kasvun estäminen on hankalaa. Permeabilisaattorit ovat yhdisteitä, jotka kykenevät vaurioittamaan Gram-negatiivisten bakteereiden ulkokalvorakennetta ja mahdollistavat hydrofobisten yhdisteiden, kuten antibioottien ja desinfektioaineiden, kulkeutumisen solun sisään, jossa ne pääsevät reagoimaan vaikutuskohteidensa kanssa.

Tässä työssä osoitettiin, että maitohappo ja lukuisat muut orgaaniset hapot (kuten bentsoe-, omena- ja sorbiinihappo, joita luontaisesti esiintyy marjoissa) heikensivät Gram-negatiivisten bakteerien ulkokalvoa. Orgaaniset hapot voivat tehostaa antimikrobisten yhdisteiden vaikutuksia. Suolistossa marjojen sisältämistä fenoliyhdisteistä muodostuvien mikrobien metaboliatuotteiden (fenyylipropioni- ja etikkahappojohdannaisia) osoitettiin heikentävän salmonellan ulkokalvoa ja herkistävän ne hydrofobisille antibiooteille. Lisäksi työssä selkiytettiin klassisen permeabilisaattorin, EDTA-kelaattorin, vaikutusmekanismeja salmonellaa kohtaan. Yhdistämällä useita permeabilisaattoriominaisuuksia mittaavia menetelmiä ja atomivoimamikroskopian antamat tulokset havaittiin, että polyetyleeni-imiini voimakkaasti vaurioitti *Pseudomonas*-bakteerien ulkokalvoa ja herkisti solut kvaternaariselle bentsalkoniumkloridi-biosidille. Yhdistämällä permeabilisaattoreiden käyttö antimikrobisiin yhdisteisiin voidaan vähentää ja laajentaa antimikrobisten yhdisteiden vaikutuksia Gram-negatiivisiin bakteereihin. Orgaanisten happojen salmonellan ulkokalvoa heikentävä vaikutus selittää osittain esimerkiksi maitohappobakteerien luontaisen kyvyn estää näiden haittamikrobien kasvua.

Preface

Studies presented in this thesis were carried out at VTT Technical Research Centre of Finland during the years 1997–2006. The research was supported by the Academy of Finland, Tekes – Finnish Funding Agency for Technology and Innovation, The European Commission and VTT.

Gram-negative bacteria are harmful in various surroundings. In the food industry their metabolites are a potential cause of spoilage and this group also includes many severe or potential pathogens, such as *Salmonella*. Due to their ability to produce biofilms, Gram-negative bacteria also cause problems in many industrial processes as well as in clinical surroundings. Control of Gram-negative bacteria is hampered by the outer membrane (OM) in the outermost layer of the cells. This layer is an intrinsic barrier for many hydrophobic agents and macromolecules. Permeabilizers are compounds that weaken OM and can thus increase the activity of antimicrobials by facilitating the entry of external substances into the cells where they can reach their target sites and inhibit or destroy cellular functions.

The Introduction aims to provide an overview of the role of Gram-negative bacteria as harmful microbes and to describe the cell structures that make them resistant against various hydrophobic agents and macromolecules. Agents capable of weakening these structures and their mechanisms are discussed. Furthermore, a brief overview of the methods used to measure weakening of the OM of Gram-negative bacteria is included. The results obtained during this study are reported in Papers I–IV.

Contents

Abstract ... 5

Tiivistelmä ... 6

Preface ... 7

List of symbols ... 10

List of publications ... 12

The author's contribution ... 13

1. Introduction ... 14
   - 1.1 Structural aspects of bacterial cell envelopes ... 14
     - 1.1.1 Gram-negative vs. Gram-positive cell ... 15
   - 1.2 Harmful Gram-negative bacteria – their importance in different surroundings ... 18
     - 1.2.1 *Salmonella* as a pathogen ... 18
     - 1.2.2 Sources of *Salmonella* ... 20
     - 1.2.3 Other Gram-negative foodborne pathogens ... 21
     - 1.2.4 Food spoilage caused by Gram-negative bacteria ... 22
     - 1.2.5 Role of Gram-negative bacteria in other environments ... 23
   - 1.3 Gram-negative cell structure ... 24
     - 1.3.1 The outer membrane ... 24
     - 1.3.2 Lipopolysaccharide ... 25
     - 1.3.3 Lipid A ... 26
     - 1.3.4 The core oligosaccharide ... 29
     - 1.3.5 The O-specific chain ... 31
   - 1.4 Permeabilizers ... 32
     - 1.4.1 Examples of permeabilizers ... 32
     - 1.4.2 Methods to detect permeabilization and weakening of Gram-negative bacterial cell membranes ... 36
   - 1.5 Factors affecting the activity of antimicrobials and permeabilizers ... 39

2. Aims of the study 41

3. Materials and methods 42

4. Results and discussion 44
   4.1 Organic acids as permeabilizers 44
      4.1.1 NPN uptake induced by organic acids 44
      4.1.2 Effect of acids on bacteriolysis 46
      4.1.3 Acids induce LPS release 46
   4.2 Effect of EDTA on the OM of *Salmonella* 49
      4.2.1 NPN uptake by cells in different growth phases 49
      4.2.2 Sensitization of bacteria to bacteriolysis induced by detergents or lysozyme 50
      4.2.3 Release of LPS 51
   4.3 Effect of colonic microbial metabolites of phenolic compounds on OM of *Salmonella* 53
      4.3.1 Effect of tested samples on the uptake of NPN 53
      4.3.2 Antibiotic susceptibility 54
      4.3.3 Release of LPS and cell lysis 54
   4.4 Weakening of environmental Gram-negative isolates with permabilizers 56
      4.4.1 Permeability assays 56
      4.4.2 Antibiotic suspectibility 58
      4.4.3 Prevention of biofilm formation on PVP plates and enhancement of *in vitro* antimicrobial activity 59
      4.4.4 Atomic force microscopy (AFM) studies 60
   4.5 Future aspects 61

5. Conclusions 64

Acknowledgements 66

References 68

Appendices

Papers I–IV

Appendix III of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://www.vtt.fi/publications/index.jsp)

List of symbols

Ac	Acetyl
AFM	Atomic force microscopy
AOT	Sodium bis-(2-ethylhexyl) sulfosuccinate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BC	Benzalkonium chloride
[^{14}C]Gal-LPS	[^{14}C]Galactose-lipopolysaccharide
CM	Cytoplasmic membrane
3,4-diHPP	3,4-dihydroxyphenylpropionic acid
DMSA	Meso-2,3-dimercaptosuccinic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Association
EPS	Extracellular polysaccharides
EtN	2-Aminoethanol
GC-MS	Gas chromatography – mass spectrometry
Hep	Heptose
HEPES	n-Heptadecanoic acid methyl ester
3-HPP	3-hydroxyphenylpropionic acid

Kdo	D-*glycero*-D-*manno*-oct-2-ulosonic acid
LPS	Lipopolysaccharide
MIC	Minimal inhibitory concentration
NPN	1-*N*-phenylnaphthylamine
NTA	Nitrilotriacetic acid
OM	Outer membrane
OMP	Outer membrane protein
PEI	Polyethylenimine
P-EtN	2-Aminoethyl phosphate
PG	Peptidoglycan
pHPP	4-hydroxyphenylpropionic acid
PMBN	Polymyxin B nonapeptide
PPEtn	Diphosphoethanolamine
RNA	Ribonucleic acid
SCWP	Secondary cell wall protein
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
S-Layer	Surface layer
TEM	Transmission electron microscopy
TPP	Tetraphenylphosphonium ion

List of publications

This thesis is based on the following original articles, which are referred in the text by their Roman numerals.

I **Alakomi, H.-L.**, Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K. & Helander, I.M. (2000). Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. Applied and Environmental Microbiology. 66, 2001–2005.

II **Alakomi, H.-L.**, Saarela, M. & Helander, I.M. (2003). Effect of EDTA on *Salmonella enterica* serovar Typhimurium involves a component not assignable to lipopolysaccharide release. Microbiology. 149, 2015–2021.

III **Alakomi, H.-L.**, Puupponen-Pimiä, R., Aura, A.-M., Helander, I.M, Nohynek, L., Oksman-Caldentey, K.-M. & Saarela, M. (In press). Weakening of *Salmonella* with selected microbial metabolites of berry-derived phenolic compounds and organic acids. Journal of Agricultural and Food Chemistry.

IV **Alakomi, H.-L.**, Paananen, A., Suihko, M.-L., Helander, I.M. & Saarela, M. (2006). Weakening effect of cell permeabilizers on Gram-negative bacteria causing biodeterioration. Applied and Environmental Microbiology. 72, 4695–4703.

The author's contribution

Paper I: Hanna-Leena Alakomi planned the experimental work together with Ilkka Helander. She performed or supervised the experimental work except the gas-chromatography measurements of fatty acid methyl esters. She was responsible for interpretation of the results and wrote the paper together with Ilkka Helander.

Paper II: Hanna-Leena Alakomi designed the experiments together with Ilkka Helander. She carried out most of the experimental work. Hanna-Leena Alakomi performed the data analysis, interpreted the results, wrote the paper together with co-authors and acted as corresponding author.

Paper III: Hanna-Leena Alakomi designed the experiments, analysed the data, interpreted the results, wrote the paper and is the corresponding author. She executed most of the experimental work, except for some cell lysis and permeability assays were carried out by technicians.

Paper IV: Hanna-Leena Alakomi planned the experimental set-up and carried out or supervised most of the experimental work. The Atomic Force Microscopy imaging and image interpretation was performed together with Arja Paananen and Hanna-Leena Alakomi analysed the image data. In addition, Hanna-Leena Alakomi analysed other data, except for 16S RNA gene sequencing data analysis which was performed by Maija-Liisa Suihko. Hanna-Leena Alakomi interpreted the results, wrote the paper and acted as corresponding author.

1. Introduction

1.1 Structural aspects of bacterial cell envelopes

Traditionally the first steps in the identification and characterisation of unknown bacteria are based on phenotypic properties of the isolate, such as colony and cell morphology. In addition, microscopic methods with various staining procedures are applied to monitor specific cell structures and to obtain information about the bacterial cell wall structures. The doublestaining method developed by Hans Christian Gram in the late 1800s is still a valuable tool in the phenotypic classification of bacteria (Holt et al., 1994, Beveridge, 2001). This method reveals differences in the cell wall structures according to which cells can be classified into two groups. Bacteria that retain the initial crystal violet stain (purple) are called Gram-positive and those that are decolorized and stain red with carbol fuchsin or safranin are called Gram-negative. Normally a reliable result is obtained from young cells, although exceptions may occur. Some bacteria, depending of their cell cycle, stain variable or stain only faintly (Beveridge, 2001). Likewise, bacteria with high membrane lipid content (e.g. mycobacteria rich in mycolic acid) require specific staining methods (Brennan & Nikaido, 1995). However, advances in 16S rRNA gene sequencing have changed the classification of some bacteria, which have a Gram-negative cell wall but are now assigned to Gram-positive phyla (Schleifer et al., 1990).

During their life microbes encounter several harsh and stressful conditions. They have to cope with changing environmental conditions, e.g. pH, temperature, salinity, antimicrobial compounds, water activity and availability of nutrients (Foster, 2004, Young, 2006). In addition, competition with other microbes for space and nutrients as well as other microbial interactions affects their life (Lazdunski et al., 2004, Young, 2006). Hence, cell membranes must be multifunctional and respond quickly to changing environmental conditions (Rowley et al., 2006).

1.1.1 Gram-negative vs. Gram-positive cell

The basic structures of Gram-negative and Gram-positive cell walls are shown in Figure 1. Cytoplasm of the cell is surrounded by the cytoplasmic membrane (CM), which is composed essentially of a phospholipid bilayer with embedded proteins (Matsumoto et al., 2006, Stenberg et al., 2005). CM is selectively permeable and regulates the transfer of solutes and metabolites in and out of the cell cytoplasm (Holland, 2004). It is also associated with several important enzymes involved in various cell metabolic functions and pumps involved in maintenance of membrane potential and ion gradients (Ruiz et al., 2006, Stenberg et al., 2005). Hence it is considered to be the major target site for biocides (Maillard, 2002). In Gram-positive bacteria CM is covered by a thick (20–80 nm) cell wall layer consisting of peptidoglycan (PG, also called murein) and adjoining polysaccharides, teichoic acids, teichuronic acids and lipoteichoic acids (Cabeen & Jacobs-Wagner, 2005, Schäffer & Messner, 2005). In addition, several Gram-positive bacteria have additional "secondary" cell wall polymers (SCWPs) which are involved in the anchoring of bacterial cell surface layers (S-layers) to the bacterial cell surface (Schäffer & Messner, 2005). PG is composed of alternating *N*-acetylglucosamine and *N*-acetylmuramic disaccharides, the latter having pentapeptide stems (Meroueh et al., 2006, Scheffers & Pinho, 2005). During cell wall biosynthesis these pentapeptide stems are cross-linked by transpeptidase enzymes, creating a cell wall polymer that is responsible for the maintenance of cell shape and osmotic stability (Daniel & Errington, 2003, Meroueh et al., 2006). The PG layer is significantly thinner (1–7 nm) in Gram-negative bacteria compared to Gram-positive bacteria (Cabeen & Jacobs-Wagner, 2005, Vollmer & Höltje, 2001). In addition, Gram-negative bacteria possess as their outermost layer a special OM that covers the periplasmic space, the PG layer and the CM (Nikaido, 2001, 2003). In Gram-negative bacteria the OM and PG are linked to each other with lipoproteins, and the OM includes porins, which allow the passage of small hydrophilic molecules (Cabeen & Jacobs-Wagner, 2005, Nikaido, 2003). In Gram-negative bacteria the periplasmic space between inner and OM store e.g. degradative enzymes (Nikaido, 2003). Recently, it has been reported that Gram-positive bacteria also have periplasmic space between CM and the mature PG (Zuber et al., 2006).

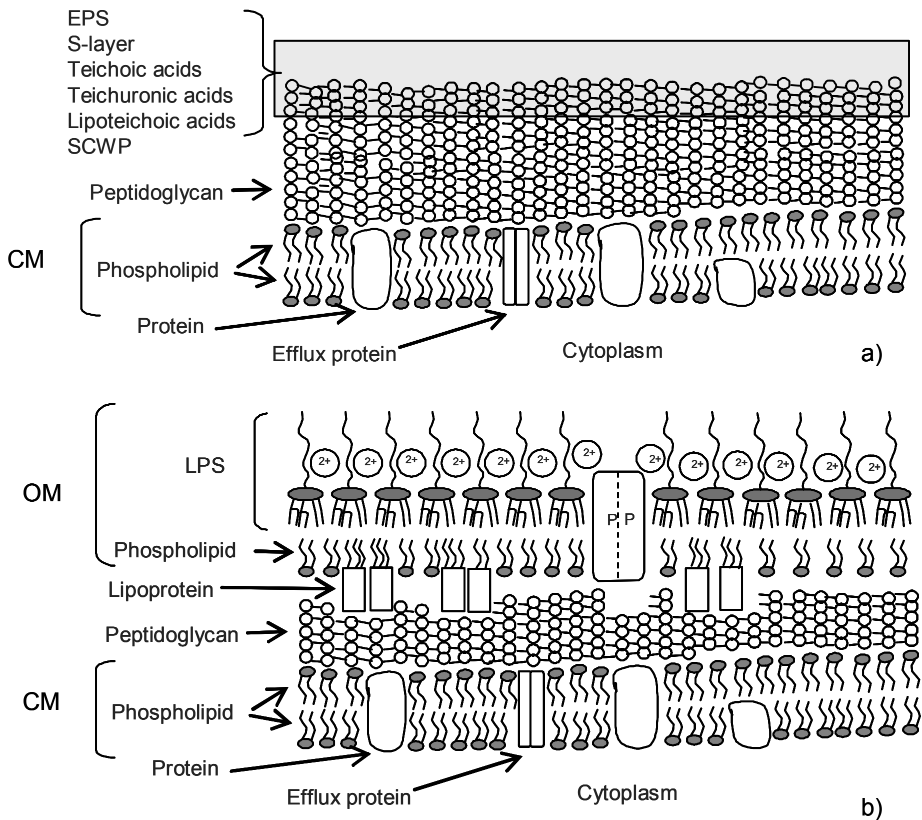


Figure 1. Cell wall architechture in a) Gram-positive bacteria and b) Gram-negative bacteria. (Modified from Schäffer & Messer [2005] and Alexander & Rietschel [2001].) In Gram-positive bacteria CM is covered by a thick cell wall layer consisting of PG and adjoining polysaccharides, teichoic acids and teichuronic acids. In addition, several Gram-positive bacteria have additional "secondary" cell wall polymers which are involved in the anchoring of bacterial cell surface layers to the bacterial cell surface. Gram-negative bacteria possess as their outermost layer a special OM that covers the periplasmic space, the PG layer and the CM. In Gram-negative bacteria the OM and PG are linked to each other with lipoproteins and the OM includes porins, which allow the passage of small hydrophilic molecules. In addition to lipopolysaccharide (LPS), some enterobacteria have enterobacterial common antigens on their OM and in some species capsular polysaccharides are also present.

The structure and composition of the outermost layer of the cells differ greatly between bacteria. On the outer envelope cells may have polysaccharide capsules (Sutherland, 1999, Snyder et al., 2006) or protein S-layers (Antikainen et al., 2002, Schäffer & Messner, 2005, Åvall-Jääskeläinen & Palva, 2005), which protect bacteria in unfavourable conditions and affect their adhesion. Bacterial membranes are responsible for many responses of the cells to antimicrobial agents and environmental stresses (Raivio, 2005). For example, the outer membrane of Gram-negative bacteria and the thick and lipid-rich cell envelope of mycobacteria act as a permeability barrier and are responsible for the intrinsic resistance of these microorganisms to antimicrobial compounds (Brennan & Nikaido, 1995, Nikaido, 2001).

Some bacteria have additional structures, such as fimbriae and pili in their outermost cell layer (Scott & Zahner, 2006). Most *Salmonella* strains produce thin aggregative fimbriae and *Escherichia coli* strains are reported to produce similar structures, termed curli (Prigent-Combaret et al., 2000, White et al., 2003). These structures in association with other components of the extracellular matrix are suggested to create a coating that affects biofilm formation and cell-cell attachment (White et al., 2001, 2003, Prigent-Combaret et al., 2000, de Rezende et al., 2005, Solano et al., 2002). For *E. coli* and *Salmonella* it has been reported that cellulose and thin aggregative fimbriae are produced together, and that they form a highly inert, hydrophobic extracellular matrix in which the cells are embedded (Zogaj et al., 2001, Solano et al., 2002, Gibson et al., 2006).

A number of harmful species and potential pathogens belong to the group of Gram-negative bacteria. The family *Enterobacteriaceae* consists of a large group of Gram-negative, non-spore forming, facultatively anaerobic bacteria (Holt et al., 1994). Some members of the *Enterobacteriaceae* family are responsible for serious infections (e.g. *Salmonella*, *Campylobacter*, *Escherichia*) in addition to more common, milder gastrointestinal infections, although they are also abundant in environments contaminated with faeces (Liebana et al., 2003, Garcia-Martin, 2004) and are part of the normal microbiota of many animals and humans (Holt et al., 1994). Since the majority of experimental work in this study was done with *Salmonella* spp., they will be discussed in more detail in the following section.

1.2 Harmful Gram-negative bacteria – their importance in different surroundings

1.2.1 *Salmonella* as a pathogen

The nomenclature of the genus *Salmonella* has been variable, since two systems of nomenclature have been in use (Tindall et al., 2005). Recently it was agreed that the genus *Salmonella* includes only two species, namely *Salmonella enterica* and *S. bongori.* The type species *Salmonella enterica* is divided into six subspecies and most *Salmonella* belong to the subspecies *Salmonella enterica* subsp. *enterica* (Tindall et al., 2005, www.bacterio.cict.fr). Members of this subspecies have usually been named based on the original isolation location of the serovar or serotype. The genus *Salmonella* is extremely heterogenous, with more than 2 500 currently recognized serovars (Popoff et al., 2004).

Salmonella, although being intestinal bacteria, are widespread in the environment and are commonly found in farm effluents and in any material subjected to faecal contamination (Le Minor, 1999, Liebana et al., 2003, Martinez-Urtaza et al., 2004). Salmonellosis is an infectious disease of humans and animals caused by living cells of the two species of *Salmonella* (*S. enterica* and *S. bongori*) (Humphrey, 2004). *S. enterica* is a facultatively intracellular pathogen which preferentially resides inside macrophages, although it requires both antibodies and a cellular immune response for clearance (Kauffman et al., 2001). Depending on the serotype and host, *Salmonella* can cause diseases ranging from mild gastroenteritis to typhoid fever (Humphrey, 2004, Chiu et al., 2004).

Salmonellosis has been reported to be the most common food-borne bacterial disease in the world (Plym Forshell & Wierup, 2006). In the United States it has been estimated that 1.4 million non-typhoidal *Salmonella* infections with 400 deaths occur annually (Voetsch et al., 2004). In 2005, salmonellosis remained the second most frequent zoonosis with about 180 000 reported human cases in the European Union (EFSA, 2006). However, it is possible that under-reporting of salmonellosis is common. *S.* Enteritidis and *S.* Typhimurium have been reported to be the serovars most frequently associated with human illness (EFSA, 2006, Olsen et al., 2002). Human *S.* Enteritis cases have often been associated with consumption of contaminated eggs (Guard-Petter, 2001, Cogan et al., 2004) and broiler meat, whereas *S.* Typhimurium cases have typically

been associated with consumption of contaminated pig, poultry and bovine meat (EFSA, 2006, Jorgensen et al., 2000). In 2005 the Finnish National Public Health Institute reported 2503 salmonella cases (Anonymous, 2006). The majority of the Finnish salmonellosis cases were caused by *S.* Enteritidis and they were obtained from abroad (Niskanen et al., 2006). *S.* Typhi remains an important health threat for mankind, with more than 16 million cases and 600 000 deaths annually world-wide (Pang et al., 1998). Typhoid fever is a disease that usually results from overcrowding and poor sanitary conditions. Hence, the incidence of this disease is highest in developing countries with poor hygienic conditions and inadequate clean water supplies and sewage systems (Gasem et al., 2002, Vollaard et al., 2004). Furthermore, the emergence of antibiotic-resistant *Salmonella* strains, e.g. due to previous uncontrolled use of antimicrobials in animal feeds and increased therapeutic use in other areas, is an increasing problem globally (Sørum & L'Abée-Lund, 2002, Su et al., 2004).

The nature of the pathogenic action of *Salmonella* varies with the serovar, the strain, the infectious dose, the nature of the contaminated food and the host status (Humphrey, 2004). Infants and immunosuppressed patients are more susceptible to salmonella infection than healthy adults (Tauxe, 2002, Le Minor 1999, Voetsch et al., 2004). Infections with *Salmonella* are initiated when the pathogen invades the gastrointestinal epithelium (Merrell & Falkow, 2004, Reynolds et al., 2006). Virulence of *Salmonella* requires multiple factors (Groisman & Ochman, 1997, Marcus et al., 2000). The cell membranes and their stress responses have an important role in virulence as most virulence determinants reside in, or must transit through them (Raivio, 2005). Lipopolysaccharide (LPS) is the major virulence factor of Gram-negative bacteria (Trent et al., 2006, Raetz & Whitfield, 2002). During the past decade, enormous progress has been achieved in the elucidation of LPS recognition and signalling in mammalian phagocytes (reviewed in Trent et al., 2006, Heumann & Roger, 2002, Reynolds et al., 2006). Recognition of LPS leads to rapid activation of an intracellular signalling pathway, which results in the release of pro-inflammatory mediators (Heumann & Roger, 2002). Pathogenic *Salmonella* bacteria have evolved many strategies in adapting to the hostile environment of the phagosome (Monack et al., 2004, Raupach & Kauffman, 2001, Reynolds et al., 2006).

Salmonella infections have been reported to result either in fatal bacteremia when unrestricted, or in the generation of neutrophil- and mononuclear-rich microabscesses that lead to bacterial clearance when successfully controlled (Merrel & Falkow, 2004). In some cases bacteria persist in the gall bladder of asymptomatic carriers, who contribute substantially to the dissemination of disease by continuous shedding of the pathogen into the environment (Raupach & Kauffman, 2001).

1.2.2 Sources of *Salmonella*

Salmonella can be associated with many kinds of food (Humphrey, 2004). Contamination of meat (cattle, pigs, poultry) may originate from animal salmonellosis, but most often it results from the contamination of meat with intestinal contents during evisceration of animals, washing, and transportation of carcasses (al-Saigh et al., 2004). Likewise, vegetables and fruits may carry salmonella if contaminated with fertilizers of faecal origin, or when washed with polluted water (Das et al., 2006, Duffy et al., 2005). O-antigen capsules produced by *Salmonella* strains potentiate their survival in the environment (Gibson et al., 2006). Although proper heat treatment of the foods will kill *Salmonella* in food, caution should be taken to avoid cross-contamination (Reij et al., 2004, van Asselt & Zwietering, 2006). Other essential elements in the prophylaxis of salmonellosis are consumer education (in particular improvement of hygiene), correct storage temperatures (preventing multiplication of *Salmonella* in food), and use of pasteurization (e.g. for milk) or sterilization whenever possible (Humphrey, 2004).

In the European Union, the Zoonoses Directive 92/117/EC requires collection of information on zoonosis and zoonotic agents in humans, animals, foods and feeds as well as monitoring of e.g. breeding flocks for *Salmonella* (EFSA, 2006). Food products of animal origin are considered to be the major source of human *Salmonella* infections and *Salmonella* infections of production animals play an important role in public health and in food safety (Plym Forshell & Wierup, 2006). Monitoring of zoonotic agents should therefore cover the whole food chain. Since feed contaminated with *Salmonella* is a potential source of contamination of farm livestock, investigation of feed and feed raw materials for *Salmonella* is also essential (Plym Forshell & Wierup, 2006, Sauli et al., 2005).

Competitive exclusion used in the poultry industry is an additional way to control *Salmonella* and has been applied successfully in Finland and Sweden for many years (Schneitz et al., 1992, Schneitz & Renney, 2003).

1.2.3 Other Gram-negative foodborne pathogens

In addition to salmonella, camplylobacteria and other Gram-negative foodborne pathogens, e.g. *Yersinia* have emerged as important pathogens (Tauxe, 2002). Due to its ability to grow at low temperatures, *Yersinia* is able to multiply during prolonged cold storage into levels high enough to cause disease (Little & Knochel, 1994). *Yersinia enterocolitica* is an important foodborne pathogen that can cause yersiniosis in humans and animals. Pigs are considered to be an important source of human yersiniosis. Similar *Yersinia* genotypes have been found among canine, feline and wild rodent strains, indicating that these animals constitute additional possible infection sources for humans (Fredriksson-Ahomaa et al., 2006).

In 2005, campylobacteriosis was the most frequently reported zoonotic disease in humans in the European Union (EFSA, 2006). Due to its ability to cause waterborne outbreaks, campylobacter epidemics normally infect a large number of people (Niskanen et al., 2006). The most important vehicle of *Campylobacter* transmission to humans is poultry meat that has been contaminated during processing (Wagenaar et al., 2006). *Escherichia coli* infections remain one of the most common causes of gastroenteritis. The majority of *E. coli* strains are harmless, but a small number can cause serious gastroenteritis (Harrington et al., 2006). *E. coli* enteritis is caused by at least six distinct *E. coli* pathotypes: enterotoxigenic, enteropathogenic, enterohemorrhagic, enteroinvasive, diffusively adherent and enteroaggregative *E. coli* (Harrington et al., 2006, Kaper et al., 2004, Venter et al., 2006).

The group of *Enterobacteriaceae* includes many other potentially opportunistic pathogens. *Enterobacter sakazakii* is an opportunistic pathogen which causes meningitis and enteritis, mostly in infants. Many of the cases have been associated with contaminated reconstituted infant formula (Drudy et al., 2006, Edelson-Mammel et al., 2006). As well as the *Enterobacteriaceae*, several other Gram-negative bacteria, such as *Aeromonas* spp. are opportunistic pathogens

(Isonhood & Drake, 2002). *Helicobacter pylori* has been reported to colonize the gastric epithelium of at least 50% of the world's human population, playing a causative role in the development of chronic gastritis and peptic ulcers (Terebiznik et al., 2006).

1.2.4 Food spoilage caused by Gram-negative bacteria

As by-products of their growth and metabolism, spoilage bacteria produce compounds (e.g. secondary metabolites) that can affect the quality of foods, causing either organoleptical, structural, chemical or microbiological changes in the products (Huis in't Veld, 1996, Gram et al., 2002). These compounds include off-flavours (e.g. volatile compounds), proteolytic and lipolytic enzymes, toxins and biogenic amines (Silla Santos, 1996, Rokka et al., 2004). In addition to enteric pathogens, the group of *Enterobacteriaceae* includes species that cause food spoilage (for reviews see Baylis, 2006). Many of the spoilage bacteria are also opportunistic pathogens able to cause disease in conditions that allow their growth. Certain members of *Hafnia*, *Serratia* and *Enterobacter* species are associated with the production of biogenic amines and off-flavours (Gram et al., 2002, Smolander et al., 2004) as well as cases of human diseases (Drudy et al., 2006).

Under aerobic conditions *Pseudomonas* spp. have been reported to be the most common spoilage microbes in foods with high protein content, such as meat, poultry, fish, milk and some dairy products (Huis in't Veld, 1996). Several members of *Pseudomonas* and *Stenotrophomonas* species produce proteolytic and lipolytic enzymes that cause spoilage of e.g. milk (Munsch-Alatossava & Alatossava, 2006). Smolander et al. (2004) reported that in modified atmosphere packed (MAP) unmarinated broiler chicken cuts stored under unbroken cold-chain conditions the spoilage was mainly caused by lactic acid bacteria, whereas in interrupted cold-chains enterobacteria and hydrogen sulphide-producing bacteria were able to multiply rapidly and produce volatile compounds and biogenic amines causing spoilage of the food (Smolander et al., 2004, Rokka et al., 2004). In addition, spoilage detected by an electronic nose (volatile compounds) correlated with the amount of *Enterobacteriaceae* in MAP products (Rajamäki et al., 2006).

In the food matrix, microbes grow in a compact ecosystem in which they can exploit metabolites produced by other microbes (metabiosis), or there may be antagonistic interactions between microbes (Fleet, 1999, Gram et al., 2002). The ability of *Salmonella* and other bacteria to survive in the food chain is partly due to their ability to respond effectively to environmental changes (Anriany et al., 2001, 2006, McMahon et al., 2007). In several cases a correlation between specific stress survival and virulence-associated phenotypes has been found (Gaynor et al., 2005). Stress-adapted cells are also more tolerant towards disinfection treatments (Loughlin et al., 2002). Contamination from process surface biofilms is one effective route for food contamination by pathogens or spoilage microbes (Ganesh Kumar & Anand, 1998, Reij et al., 2004).

1.2.5 Role of Gram-negative bacteria in other environments

In nature and in process environments microbes grow as multi-species communities (biofilms) attached to surfaces where they are protected from the action of biocides and disinfectant agents (Davey & O'Toole, 2000, Guerrero et al., 2002). Cells of the community have close interactions with each other (multiple attachments, cell-cell signalling) and they cooperate for obtaining nutrients and metabolic compounds (Davey, 2000, Young, 2006). In addition, cells in biofilm exhibit an altered phenotype (e.g. slower growth rate and gene expression) compared to free-swimming (planktonic) cells (Young, 2006). Biofilm structure provides the cells with protection from an exposure to external agents (e.g. biocides, antibiotics) (Campanag et al., 2002, Donlan & Costerton, 2002, Snyder et al., 2006). In particular, extracellular polysaccharides (EPS) play various roles in the structure formation and function of different biofilm communities: they exclude and/or influence the penetration of antimicrobial agents, and provide protection against a variety of environmental stresses such as UV radiation, pH shifts, osmotic shock, and desiccation (Campanag et al., 2002, Davey & O'Toole, 2000, Robertson & Firestone, 1992, Young, 2006). In addition, polysaccharide capsules present on bacterial surfaces strengthen interactions between microbes and biofilm matrix (Sutherland, 1999, Snyder et al., 2006). Since Gram-negative bacteria are efficient producers of extracellular matrix and polysaccharide capsules, they play an important role in the formation and stabilization of biofilm structures on surfaces (Zogaj et al., 2001, Gibson et al., 2006, White et al., 2006). Schooling and Beveridge (2006) recently reported

that membrane vesicles produced by *Pseudomonas* sp. are an important part of biofilm structures.

Pseudomonas sp. biofilms are important in clinical environments (e.g. on the surfaces of medical devices) (Donlan & Costerton, 2002). Likewise they cause problems in various industrial systems e.g. in the food processing and paper processing industries (Lahtinen et al., 2006, Wirtanen et al., 2001).

In outdoor environments the microbial mats are composed of complex microbial ecosystems, whose members have adapted to extreme environmental conditions (Walker et al., 2005). The endolithic environment, the pore space of rocks, has been reported to be a microhabitat providing protection from intense solar radiation and desiccation, as well as supplying mineral nutrients, rock moisture and growth surface (Walker et al., 2005). The phototrophs, algae and cyanobacteria have been considered to be the primary colonizers of building surfaces, conditioning the surfaces and secreting nutrients and growth factors for heterotrophic microbes (Crispim & Gaylarde, 2005). Besides phototrophic cyanobacteria, many other Gram-negative bacterial species, e.g. members of the genera *Pseudomonas*, *Stenotrophomonas* and *Sinorhizobium*, have been isolated from biodeteriorated stone samples (Crispim & Gaylarde, 2005, Saarela et al., 2004). In addition to aesthetic damage, organisms present on stone monuments such as chemolithoautotrophic and heterotrophic bacteria produce metabolites that are able to deteriorate stone surfaces (Crispim & Gaylarde, 2005, Dornieden et al., 2000, Warscheid & Braams, 2000).

1.3 Gram-negative cell structure

1.3.1 The outer membrane

Restriction of growth of Gram-negative bacteria is hampered by their relative resistance to many external agents (Nikaido, 1999). Cells of Gram-negative bacteria are surrounded by an additional membrane (outer membrane, OM), which provides the bacterium with a hydrophilic surface and functions as a permeability barrier for many external agents (Helander et al., 1997c, Vaara, 1992, 1999, Nikaido, 2003). The effect is mainly caused by the presence and features of LPS molecules in the outer leaflet of the membrane, resulting in an

inherent resistance to hydrophobic antibiotics, detergents and hydrophobic dyes in many Gram-negative bacteria (Nikaido & Vaara, 1985, Nikaido, 2003). The OM of common Gram-negative bacteria, such as *E. coli*, is constructed asymmetrically (Beveridge, 2001). The lipid structure of the membrane's outer leaflet is formed dominantly by lipid A regions of the LPS molecules, which make up 75% of the total membrane surface and form specific contacts with integral outer membrane proteins (Omp), such as porins (Alexander & Rietschel, 2001, Bos & Tommassen, 2004). Bacterial lipoproteins anchor the OM to the periplasmic peptidoglycan layer (Bos & Tommassen, 2004). Divalent cations are tightly associated with the anionic membrane-proximal regions of LPS molecules, strengthening the structure (Vaara, 1992). Some Gram-negative bacteria are known to contain glycosphingolipids instead of LPS in their OM (Kawahara et al., 1991). In addition to LPS, some enterobacteria have present on their OM surface an additional glycolipid, the enterobacterial common antigen (Erbel et al., 2003, Rick & Silver, 1996) and some species and strains also have capsular polysaccharides on their cell surface (Snyder et al., 2006).

Gram-negative bacteria regulate OM permeability characteristics with hydrophilic channels known as porins, which allow nutrients with relatively small molecular weight (< 600 Daltons) to enter the inner parts of the cell (Nikaido, 2003). These water-filled pores generally exclude the entry of hydrophobic substances (Nikaido, 2003). The influx of lipophilic compounds into gram-negative cells is limited not only by the OM permeability barrier but also by their active efflux by "multidrug" efflux pumps, which are usually energized by the proton motive force (Poole, 2002, Piddock, 2006). *Pseudomonas* species have been reported to be resistant to many biocides and antimicrobial agents (Poole, 2002, Walsh et al., 2000).

The components of the OM are synthesized inside the cell or at the inner leaflet of CM (Wu et al., 2006, Ruiz et al., 2006). During recent years there has been a marked advance in the identification of the steps of bacterial OM biosynthesis (Raetz & Whitfield, 2002, Bos & Tommassen, 2004, Ruiz et al., 2006, Trent et al., 2006).

1.3.2 Lipopolysaccharide

LPS molecules localized on the outer leaflet of the outer membrane constitute the major surface component of the Gram-negative bacterial cell envelope

(Nikaido, 1999, Trent et al., 2006). This glycolipid is essential for virtually all Gram-negative bacteria and represents one of the conserved microbial structures responsible for activation of the innate immune system (Reynolds et al., 2006). Therefore the structure, function, and biosynthesis of LPS have been an area of intense research (recently reviewed by Alexander & Rietschel, 2001, Trent et al., 2006). LPS research started with *E. coli* and *Salmonella*, but nowadays the structures and biosynthesis of many other Gram-negative bacteria are also well known (Raetz & Whitfield, 2002). LPS is typically composed of three distinct regions: lipid A (endotoxic principle and anchoring molecule in the OM), a core oligosaccharide, and O-antigenic polysaccharide (highly variable O-antigenic polysaccharide consisting of repeating units) (Alexander & Rietschel, 2001). The basic structure of LPS, a covalently linked lipid and heteropolysaccharide (Figure 2), is common to all LPS molecules studied, but otherwise there are extensive variations in the chemical structures of LPS depending on bacterial genera, species and strains (for a review see Trent et al., 2006, Raetz & Whitfield, 2002, Yethon & Whitfield, 2001a, 2001b).

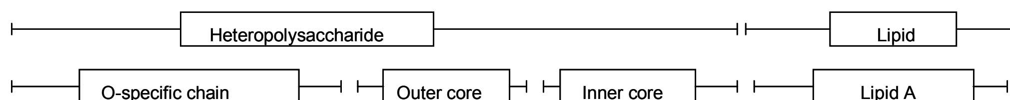


Figure 2. General structure of LPS.

1.3.3 Lipid A

The lipid A domain anchors the LPS in the outer membrane and is the bioactive component recognized by Toll Like Receptor 4 during human infection (primary immunostimulatory centre of LPS) (Trent et al., 2006). In human LPS-responsive cells, the phosphate groups and the secondary myristoyl and lauroyl residues (the length and number of fatty acyl chains) of enterobacterial type lipid A play a key role in the specific recognition and activation of innate immunity (Alexander & Rietschel, 2001, Trent et al., 2006). In severe cases lipid A can stimulate overproduction of tissue factors, leading to lethal sepsis or septic shock (Alexander & Rietschel, 2001). A common type of lipid A occurs in *E. coli*, in which the hydrophilic backbone consists of a β-(1→6)-linked 2-amino-2-deoxyglucopyranose (GlcN) disaccharide, carrying two phosphate groups at

positions 1 and 4’ and four residues of (R)-3-hydroxymyristic acid in ester and amide linkages (Zähringer et al., 1994, Gronow & Brade, 2001). The hydroxyl groups of some of the fatty acids are further esterified by nonhydroxyl fatty acids, creating the unique 3-acyloxylacyl structure (Helander et al., 1996, Figure 3). Negatively charged phosphate groups are important for strengthening the LPS monolayer by linking molecules via ionic bridges with divalent cations (Vaara, 1999). The phosphate groups of the lipid A backbone may carry additional substituents such as 2-aminoethanol (EtN), 2-aminoethyl phosphate (P-EtN) and 4-amino-4-deoxy-L-arabinopyranose (L-Ara*p*4N) (Helander et al., 1996).

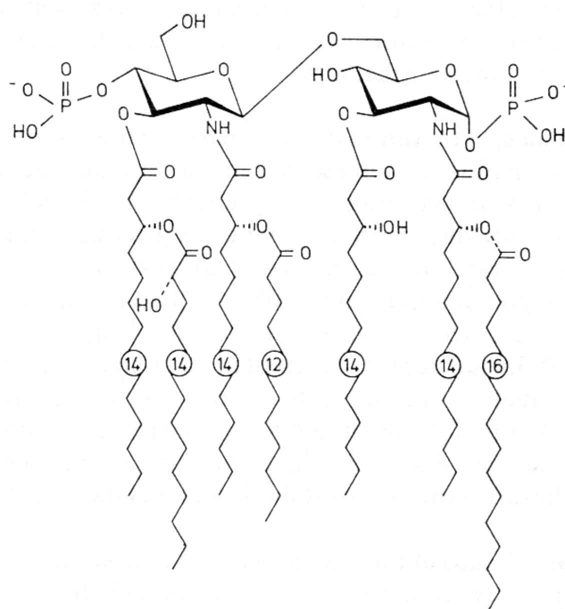


Figure 3. Chemical structure of lipid A in Salmonella minnesota mutant strain R595. Source: Zähringer et al. (1994).

Although the lipid A structures of various organisms exhibit some level of diversity, the biochemical synthesis of lipid A is considered to be a highly conserved process (Raetz & Whitfield, 2002, Reynolds et al., 2006). Recent studies have shown that *S.* Typhimurium cells can respond to certain environmental conditions by producing enzymes that alter the lipid A structure, thereby changing e.g. membrane fluidity (Reynolds et al., 2006, Venter et al., 2006). Lipid A modifications are primarily involved in survival in environments

with low levels of divalent cations, in providing resistance to cationic peptides, in growth at low temperatures and in virulence (Guo et al., 1998, Frirdich & Whitfield, 2005, Gutsmann et al., 2005, Reynolds et al., 2006). In *E. coli* and *Salmonella* the attachment of phosphoethanolamine and L-Ara4N moieties is also induced by exposure to mildly acidic conditions, or by mutation (Helander et al., 1994, Nummila et al., 1995, Raetz & Whitfield, 2002). The presence of the L-Ara4N substituent protects bacteria against killing by polymyxin and certain antibacterial peptides (Gunn et al., 1998, Gunn, 2001). The Ara4N addition creates a more positively charged LPS and thus reduces cationic antimicrobial peptide binding (Gunn et al., 2000). Members of the genus *Yersinia* have also been reported to be able to modify their lipid A structure in response to temperature changes (Bengoechea et al., 2003, Rebell et al., 2004). Lipid A modifications require the activation of the two-component regulatory system PhoP-PhoQ, which is essential for virulence of *Salmonella* (Guo et al., 1997).

Lipid A is generally required for bacterial growth as it is needed to maintain the integrity of the outer membrane barrier (Vaara, 1992). Therefore, several inhibitory agents targeting its synthesis or enzymes of the biosynthesis, such as LpxC, have been investigated for the production of new antimicrobials (Jackman et al., 2000, Yethon & Whitfield, 2001a, Mdluli et al., 2006). However, certain strains of *Neisseria meningitis*, which have a polysialic acid capsule, have been reported to be able to grow slowly even without lipid A (Steeghs et al., 1998). A representative structure of LPS, in this case that of *Salmonella*, is shown in Figure 4.

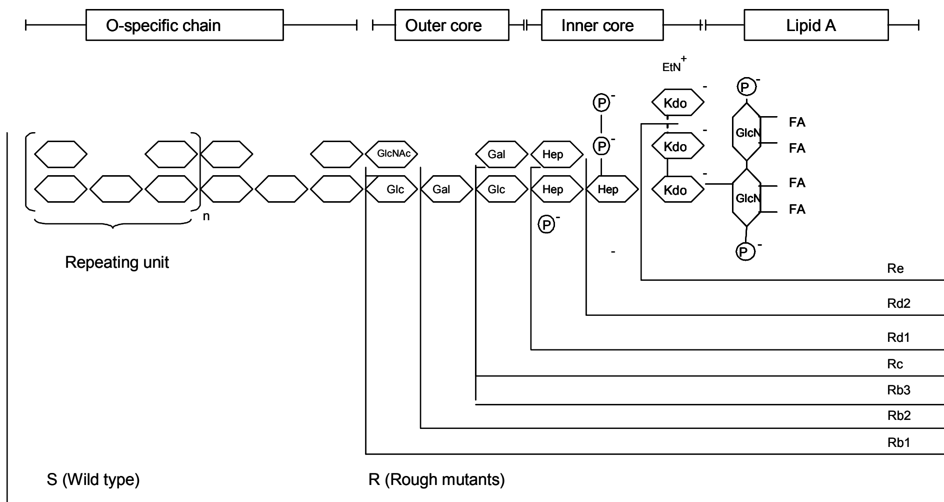


Figure 4. General structure of LPS from Salmonella. Abbreviations of monosaccharide residues: GlcN, glucosamine; Kdo, 2-keto-3-deoxyoctulosonic acid (3-deoxy-D-manno-octulosonic acid); Hep, L-glycero-D-manno-heptose; FA, fatty acid; P, phosphate, EtN, data according to Alexander & Rietschel (2001) and Helander et al. (1996). The structure of the Ra chemotype core oligosaccharide is presented in this picture. In the second core type of Salmonella the terminal D-GlcNAc residue present in the Ra chemotype is replaced by a D-Glc residue (Olsthoorn et al., 1998). Furthermore, phosphoryl substituents attached to O-4 of Hep I and II were indentified as 2-aminoethyl diphosphate and phosphate, respectively (Olsthoorn et al., 1998).

1.3.4 The core oligosaccharide

The complex heteropolysaccharide (core oligosaccharide and O-specific chain) is covalently linked to position 6' of lipid A (Gronow & Brade, 2001). The core region can be further subdivided on the basis of structural features into two regions, the inner and outer core. The outer core typically consists of common hexose sugars, such as glucose, galactose, *N*-acetyl galactosamine and *N*-acetyl glucosamine and is generally more variable than the inner core region (Holst, 1999, Erridge et al., 2002). The inner core region is composed of at least one molecule of 3-deoxy-α-D-*manno*-oct-2-ulopyranosonic acid (often also called 2-keto-3-deoxyoctulosonic acid, Kdo) and two or more residues of L-*glycero*-α-

D-*manno*-heptopyranose (L,D-Hep) (Alexander & Rietschel, 2001, Frirdich & Whitfield, 2005). Often additional anionic substituents such as phosphate, diphosphate or diphosphoethanolamine (PPEtn) are linked to the core structure (Raetz & Whitfield, 2002). The structure of the inner core tends to be conserved within a genus or family (Raetz, 1996, Raetz & Whitfield, 2002). For example, for *Salmonella* two types of core structures are currently known (Olsthoorn et al., 1998). LPS molecules extracted from a given isolate exhibit variation in the extent of core completion (Raetz & Whitfield, 2002). According to Yethon et al. (1998), modification of the core region of *E. coli* and *Salmonella* LPS requires the involvement of three enzymes, WaaP (an LPS kinase), WaaY (an enzyme required for a secondary phosphorylation) and WaaQ (a transferase that adds the side-branch heptose). Various studies performed with rough mutant strains (lacking the O-antigen and in some cases part of the core due to a genetic defect, Figure 4) have advanced the understanding of the structure and importance of the bacterial core region (Vaara, 1992, Helander et al., 1994, 1997b). The mutants with a complete R core (Ra LPS) or with a core defecient only in the galactose and *N*-acetylglucosamine moieties (Rb LPS) exhibit an essentially unaltered permeability barrier (Nikaido, 2003).

LPS consisting of Lipid A and Kdo with a negatively charged substituent is the smallest known structure to sustain growth and viability of Gram-negative bacteria (Helander et al., 1988), except for some *Neisseria* species which have been shown to be viable without LPS (Steeghs et al., 1998, Bos & Tommassen, 2005). The negative charges provided by the phosphate residues in the Hep region of *E. coli* and *Salmonella* are important in maintaining the barrier function of the OM by providing sites for cross-linking of adjacent LPS molecules with divalent cations or polyamines (Vaara, 1992, Frirdich & Whitfield, 2005). These negative charges, provided by residues of Kdo and phosphate, allow neighbouring LPS molecules to be crosslinked by divalent cations (Mg^{2+}, Ca^{2+}), structurally reinforcing the OM (Nikaido, 2001). Likewise, the negative charges play an important role in creating interactions between LPS and positive charges of OM proteins (OMPs). This part of the LPS has been found to be selectively targeted by several cationic antibiotics, positively charged host defence peptides and proteins such as the family of mammalian defensins (Alexander & Rietschel, 2001). According to Walsh et al. (2000) the inner core phosphates of *P. aeruginosa* appear to play a key role in the intrinsic drug resistance of this bacterium. In addition, ethanolamine in the core of

P. aeruginosa has been suggested to play a role in conferring resistance to cationic antimicrobial peptides (Knirel et al., 2006). On the other hand, *P. aeruginosa* has been reported to be highly susceptible to lysis with EDTA due to its extensively phosphorylated inner-core heptose region (Kooistra et al., 2003).

Klebsiella pneumoniae strains typically express smooth LPS with O-antigen polysaccharide and antigenic capsular polysaccharide on their surface, which contribute to the pathogenesis of the species (Fresno et al., 2006). One of the features of the *K. pneumoniae* core oligosaccharides is the absence of phosphate residues (Severn et al., 1996), but they contain galacturonic acid residues, which contribute to the negative charges (Fresno et al., 2006). The negative charges provided by the carboxyl groups of galacturonic acid play an important role in capsule attachment by an ionic interaction (Fresno et al., 2006, 2007). Likewise, *Rhizobium etli* LPS core region differs substantially from the typical enterobacterial cores (Forsberg & Carlson, 1998). *R. etli* strains lack both heptose and phosphate, and galacturonosyl residues provide negative charges that stabilize the core structure (Forsberg & Carlson, 1998).

1.3.5 The O-specific chain

Many pathogenic bacteria have in their LPS an O-specific (O-antigen) chain which provides an additional shelter against environmental factors and the host's defence mechanisms (Alexander & Rietschel, 2001, Szacfranek et al., 2006, Venter et al., 2006). The O-specific chain consists of up to 50 repeating units of mono- or oligosaccharides and is highly variable in structure even within a single bacterial species (Parker et al., 2001, Gronow & Brade, 2001, Szafranek et al., 2006). The chain length heterogeneity of LPS populations is observed in SDS-PAGE analysis of LPS samples, where they appear as a "ladder" in stained SDS-PAGE gels (see for example Figure 1 in Paper I, Hitchcock & Brown, [1983]). The presence of O-antigen results in a "smooth" colony appearance (S-form LPS) in contrast to the "rough" morphology displayed by colonies of O-antigen-deficient bacteria (R-form LPS) (Helander et al., 1996). As predominant surface structures O-antigens are highly immunogenic (Gronow & Brade, 2001). Accordingly, O-specific antibodies are versatile tools for serotyping (Popoff et al., 2004). In bacterial cells O-antigens are produced by similar pathways as for capsular and exopolysaccharides, but for LPS

biosynthesis they involve additional ligation steps for the assembly to lipid A-core structure as well as a translocation machinery to translocate the completed S-LPS to the surface of OM (Raetz & Whitfield, 2002).

The O-antigen is generally not essential for the survival of bacteria, but several studies have shown that O-antigen plays an important role in the effective colonization of host tissues, resistance to complement-mediated killing and in the resistance to cationic antimicrobial peptides that are key elements of the immune system (Skurnik & Bengoechea, 2003). These phenomena have been reported e.g. for *Yersinia* (Skurnik & Bengoechea, 2003) as well as for *Salmonella* (Guard-Bouldin et al., 2004, Carroll et al., 2004). Certain mucosal pathogens (e.g. some *Campylobacter jejuni* and *Neisseria* strains) have been shown to produce low molecular weight LPS (or lipo-oligosaccharide) and lack the specific O-chain in their LPS (Preston et al., 1996).

1.4 Permeabilizers

Although the OM of Gram-negative bacteria protects cells from many external agents, it is possible to weaken it specifically by various agents, collectively called permeabilizers, which disintegrate the LPS layer and increase the permeability of the OM to hydrophopic agents (Vaara, 1992, 1999). Permeabilizers themselves may not be bacteriocidal, but they may potentiate the activity of other compounds, thus acting synergistically (Vaara, 1999). Some researchers have also used mechanical means, e.g. high hydrostatic pressure, for mechanical disturbance of OM and thereby observed increased sensitivity to lysozyme in Gram-negative bacteria (Nakimbugwe et al., 2006). However, these mechanical methods are not included in this discussion.

1.4.1 Examples of permeabilizers

The classical example of permeabilizers is the chelator EDTA, which sequesters divalent cations that contribute to the stability of the OM by providing electrostatic interactions with proteins and LPS (Leive, 1965, Vaara, 1981, 1999). Treatment with EDTA releases a large proportion of LPS from the OM, exposing hydrophobic phospholipids and creating a hydrophobic pathway for

certain substances (Leive, 1965). This is noticeable as an increased susceptibility to hydrophobic agents (Hancock, 1998, Helander et al., 1997c, Nikaido, 1996a, Vaara, 1999). Figure 5 shows a schematic view of the activity of EDTA. EDTA has been reported to potentiate the activity of cell wall degrading agents (e.g. lysozyme, nisin) and biocides against microbes (Helander et al., 1997c, Shelef & Seiter, 1993, Vaara, 1981, Walsh et al., 2003a, 2003b). In addition, EDTA may act as a direct inhibitor of several species of bacteria or it may act synergistically with other antimicrobial agents to promote bacterial destruction (Shelef & Seiter, 1993). Besides EDTA, a number of other permeabilizers are known, some of which act quite differently (Helander et al., 1997a, Vaara, 1992). Table 1 summarises examples of known permabilizers and their known mechanisms of action against Gram-negative bacteria.

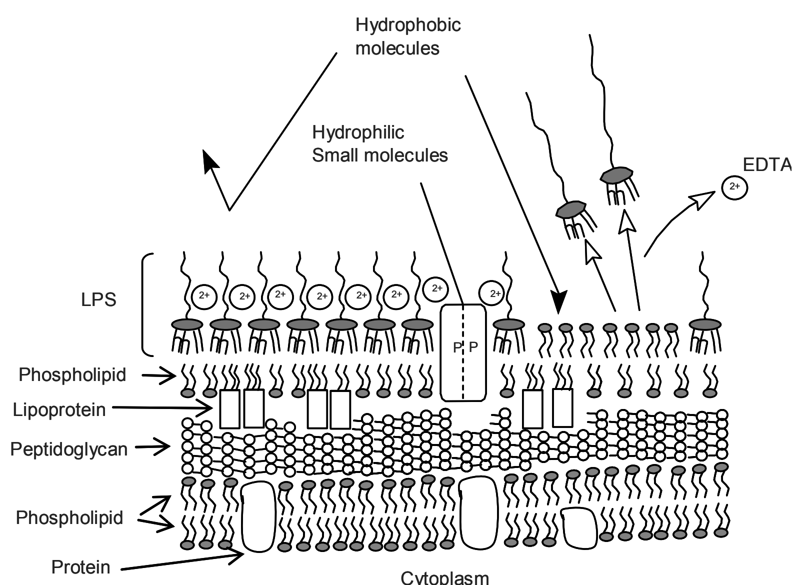


Figure 5. Permeability barrier function and disruption of the OM. The intact outer membrane (left-hand side of the figure) functions as a permeability barrier against hydrophobic molecules, but allows the entry of small hydrophilic molecules through porin proteins (PP). Removal of stabilizing divalent cations (2+) from the lipopolysaccharide (LPS) layer by chelating agents such as EDTA results in the release of LPS and appearance of phospholipids on the surface of the outer membrane (right-hand side of the figure), whereby hydrophobic compounds can penetrate the outer membrane and reach their site of action at the cytoplasmic membrane (modified from Helander et al., 1997c).

Table 1. Examples of permeabilizers and other substances possessing OM-disrupting activity and their mode of action against Gram-negative bacteria.

Agent	Mode of action	References
Chelators		
Ethylenediaminetetraacetic acid	Removes stabilizing cations from the OM, notably Ca^{2+} and Mg^{2+}. Releases LPS to the external medium and creates a hydrophobic pathway.	Finch & Brown (1975), Leive (1965, 1974), Helander et al. (1997c), Shelef & Seiter (1993), Vaara, (1992, 1999), Walsh et al. (2003b)
Na-hexametaphosphate	Removes stabilizing cations from the OM, notably Ca^{2+} and Mg^{2}. Increases sensitivity to hydrophobic antibiotics.	Vaara & Jaakola (1989)
Na_2-pyrophosphate, Na-orthophosphate	Destabilises OM. Sensitizes cells to nisin.	Boziaris & Adams (1999)
Nitrilotriacetic acid	Disintegrates the OM. Increases sensitivity to hydrophobic antibiotics.	Hancock & Wong (1984), Ayres et al. (1998)
Polycationic agents		
Polymyxins	Displaces cations from the OM, causes membrane damage.	Nikaido & Vaara (1985), Hancock & Wong (1984)
Tris (high concentrations)	Binds to OM and increases sensitivity to hydrophobic antibiotics.	Nikaido (1996a, b)
Polymyxin B nonapeptide	Permeabilizes the OM without significant release of LPS. Increases the cell surface hydrophobicity.	Vaara & Vaara (1983 a, b)
Poly-L-ornithine, Poly-L-lysine	Permeabilises the OM to hydrophobic antibiotics and releases LPS.	Hancock & Wong (1984), Vaara & Vaara (1983a, b)
L-Ascorbate, Acetylsalicylate	Destabilizes the OM.	Hancock & Wong (1984)
Lactoferrin, transferrin	Releases LPS, increases sensitivity to rifampin.	Ellison et al. (1988)
Cationic detergents, e.g. benzalkonium chloride	Destabilizes hydrophobic interactions in OM.	Hancock & Wong (1984)

Polyethyleneimine	Intercalates in the OM and increases the membrane surface area without liberation of LPS-associated cell material. Sensitizes target cells to hydrophobic antibiotics and to detergents; causes the formation of vesicular structures on the surface of OM.	Helander et al. (1997a, 1998b)
Membrane-perturbing proteins and peptides		
Synthetic cationic peptides	Disorganization of LPS by interaction of the peptide with the anionic and hydrophobic lipid A.	Vaara & Porro (1996)
Cationic amphiphilic peptides		Wiese et al. (2003)
Terpenoid and phenolic compounds found in berries and herb plants		
Thymol, carvacrol	Destabilizes the OM and causes LPS release.	Helander et al. (1998a)
Gallic acid	Displaces cations from the OM, causes membrane damage and LPS release.	Nohynek et al. (2006)
Phenolic berry extracts (cloudberry and raspberry)	Displaces cations from the OM, causes membrane damage and LPS release.	Nohynek et al. (2006)
Other compounds		
Chitosan (polymeric β-1,4-*N*-acetylglucosamine	Binds to OM resulting in the loss of barrier function.	Helander et al. (2001)
Quinolones	Low amounts (0.25 x MIC) of quinolones increases the sensitivity of Gram-negative bacteria to antimicrobial peptides by interacting with the OM by removal of stabilizing divalent cations from LPS-binding sites.	Campos et al. (2006)
Organic acids and their salts		
Citric acid	Chelates cations from the OM, notably Ca^{2+} and Mg^{2+}, induces release of LPS.	Helander & Mattila-Sandholm (2000)
Succinate, acetate, citrate	Weakly increases membrane permeability.	Hancock & Wong (1984)

Microbicidal acids belong to the membrane-active substances (Paulus, 1993). A major part of the inhibitory effects of weak acids used as food preservatives are related to the undissociated forms of acids which can penetrate cell membranes, causing perturbation of membrane function, acidification of the cytoplasm and inhibition of acid-sensitive enzymes (Hirshfield et al., 2003). The undissociated forms of acids can interact with or pass through the membrane of the microbial cell, which is normally negatively charged, and serves as a barrier to the negatively charged forms of acids (Doores, 1993). Weak organic acids, e.g. citric acid have been reported to permeabilize Gram-negative bacteria (Helander & Mattila-Sandholm, 2000). Table 2 summarizes the chemical structure and pKa values of selected organic acids.

Table 2. Chemical structure and pKa values of selected organic acids. Data modified from (Doores, 1993).

Compound (systematic name)	**Chemical structure**	**pKa at 25 °C**
Benzoic acid (benzenecarboxylic acid)	C_6H_5COOH	4.19
Citric acid	$HOC(COOH)(CH_2COOH)_2$	3.13
Malic acid (DL-hydroxybutanedioic acid)	$HO_2CCH_2CH(OH)CO_2H$	3.40
Lactic acid (2-hydroxypropionic acid)	$CH_3CH(OH)COOH$	3.86
Sorbic acid (2,4-hexadienoic acid)	$CH_3CH{=}CHCH{=}CHCOOH$	4.76

1.4.2 Methods to detect permeabilization and weakening of Gram-negative bacterial cell membranes

Several microbiological, chemical, chromatographic and fluorometric assays are used for the assessment of Gram-negative bacterial permeabilization. The permeabilizing activity of a certain compound against Gram-negative bacteria can be assessed by measuring the sensitization of bacteria to hydrophobic agents, e.g. antibiotics, dyes and other hydrophobic compounds by the agar

diffusion method (Vaara, 1981) or in liquid cultures by monitoring growth inhibition (Ayres et al., 1998, Boziaris & Adams, 1999, Bengoechea et al., 2003). Measurement of an increase of the uptake of a hydrophopic probe (fluorochromes) is another widely applied method to monitor OM permeability. This is based on the use of fluorochromes, such as 1-*N*-phenylnaphthylamine (NPN), which are non-fluorescent in aquatic environments but become fluorescent when in contact with lipids (Träuble & Overath, 1973, Hancock & Wong, 1984, Loh et al., 1984, Bengoechea et al., 1998, Helander & Mattila-Sandholm, 2000). In addition, sensitization of Gram-negative cells to cell lysis induced by detergents (e.g. sodium dodecyl sulphate [SDS] and Triton X-100) as well as by lysozyme and deoxycholate are indications of weakening of the OM (Vaara, 1981, Ayres et al., 1993, Helander et al., 1997a, Bengoechea et al., 1996, 1998). According to Yasudu et al. (2003) an increase in the uptake of tetraphenylphosphonium ion (TPP+, a hydrophobic lipophilic ion) could be monitored from cells with a distrupted OM permeability barrier.

There is evidence that some bacteria use efflux pumps to force out hydrophobic compounds, thereby complementing the barrier function of OM (Nikaido, 2003). Since efflux systems use energy, in some cases it can be useful to run permeability assays in the presense of metabolic inhibitors (e.g. potassium cyanide, sodium arsenite) that prevent efflux by de-energizing the cells (Bengoechea et al., 1998, Skurnik et al., 1999).

In addition, destabilization of the OM can be detected by measuring the release of OM-specific compounds, e.g. LPS, by monitoring LPS-specific fatty acids (Vaara, 1981, 1992, Helander et al., 1998a) or radiolabelled LPS compounds from cell-free supernatants of treated cells (Hukari et al., 1986) as well as other membrane-specific material, e.g. phospholipids. A traditional way is to run SDS-PAGE gels to reveal LPS released in cell-free culture supernatants of treated cells (Hitchcock & Brown, 1983). Chemical characterization of the released LPS fractions e.g. by ^{31}P-NMR (Helander et al., 1997b) reveals possible differences in the LPS. However, a number of permeabilizers are known not to release LPS, e.g. polyethylenimine (PEI) (Helander et al., 1997a, 1998b) and polymyxin B nonapeptide (PMBN) (Vaara, 1992).

Detection of cytoplasmic membrane damage in Gram-negative cells indicates massive destabilization of OM. In studies of combined and synergistic uses of

permeabilizers and antimicrobial agents, measurement of the CM permeabilization is useful. Leakage of low molecular weight cytoplasmic compounds from the cell interior can be used as an indicator of disorganization of the cytoplasmic membrane (Johnston et al., 2003). Potassium ion-sensitive electrodes, flame photometry and inductively coupled plasma spectrometry have been used to detect K+ leakage from cells after exposure to antimicrobials (Orlov et al., 2002, Yasudu et al., 2003, Johnston et al., 2003). Ohmizo et al. (2004) combined a TPP+-selective electrode and K+ electrode to monitor changes in the cytoplasmic and in the outer membrane permeability simultaneously. In permeabilized cells the leakage of low molecular weight compounds is frequently followed by leakage of larger cellular constituents (e.g. ATP) or efflux of A_{260nm} absorbing material (Chen & Cooper, 2002). Changes in membrane depolarization can be measured by using fluorochromes such as 3,3'-dipropylthiocarocyanide, which is taken up by bacterial cells according to their membrane potential and concentrates in the CM, where it quenches its own fluorescence. Agents that permeabilize the CM depolarize the membrane potential and cause release of the fluorochrome, which results in an increase in the fluoresecence (Wu & Hancock, 1999). El-Kosasy (2006) recently introduced a potentiometric method for the assessment of Gram-negative bacteria permeability towards some antibiotics, e.g. tobramycin. With Gram-negative bacteria the cytoplasmic β-galactosidase activity (monitored with a chromogenic substrate, O-nitrophenyl-β-D-galactopyranoside) can be used to monitor the level of permeabilization (Silvestro et al., 2000). Likewise, hydrolysis of the chromogenic β-lactam nitrocefin by periplasmic β-lactamase has been used to assess the effects of permeabilizers on the OM permeability (Hancock & Wong, 1984).

Several researchers have also constructed membrane vesicles to mimic bacterial and mammal membranes (Glukhov et al., 2005). These have been widely applied to study the efficacy of cationic antimicrobial peptides, e.g. defensins and polymyxin B (Wiese et al., 2003). However, many of the experiments performed with cationic peptides of animal origin (such as defensins) have been carried out by using buffers of low ionic strength (Nikaido, 2003). Therefore, their OM-weakening activity in higher ionic concentrations (e.g. 0.1 M NaCl, mimicking physiological conditions in the human body) may be lower (Nikaido, 2003).

Microscopical methods, such as Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM), are useful tools in monitoring bacterial membrane structures and microbial samples. However, these methods require multistep pretreatments (e.g. dehydration) of the sample, which may alter fragile membrane structures (Holt & Beveridge, 1982). Recent developments in Atomic Force Microscopy (AFM) techniques are providing new ways to examine bacteria without extensive pretreatments (recently reviewed by Ubbink & Schär-Zammaretti, 2005). With AFM it is also possible to measure forces involved in bacterial interactions and attachment on surfaces (Gaboriaud et al., 2005, Schär-Zammaretti & Ubbink, 2003). In AFM bacteria retain their cell shape and membranes intact (Bolshakova et al., 2004), allowing detailed inspection of various membrane structures, such as porins (Dufrene, 2001, Gaboriaud et al., 2005). In addition, AFM has been applied to study the effects of antimicrobials, e.g. antibiotics on the surface of bacteria (Meincken et al., 2005). Comparison of results obtained with various microscopic (e.g. TEM and AFM) and biophysical techniques provide a wider view of the function and structure of the cell (Schär-Zammaretti & Ubbink, 2003, Ubbink & Schär-Zammaretti, 2005).

1.5 Factors affecting the activity of antimicrobials and permeabilizers

The efficacy of antimicrobial agents in various matrices is influenced by many factors. Among the most important effects are the physiological parameters of the target microbe (e.g. hydrophobicity of the cell surface and cell membrane structures) as well as the previous adaptation of the target cells to stressful conditions (Brul et al., 2002). The composition of the matrix where the antimicrobial agent should be active, e.g. the amount of divalent cations or lipids in food, is an additional factor, as many antimicrobial compounds can react with them and thereby the activities of several antimicrobials may be significantly decreased (Davidson, 1997, Scotter & Castle, 2004).

The incidence of multi-drug resistant bacteria and the emergence of new and opportunistic pathogens continuously set new demands for more effective means for pathogen control (Levy, 2002). Reports of reduced susceptibility of microbes to various biocides are also increasing (Walsh et al., 2003a). The basic mechanisms of antibiotics are generally well documented (Russell, 2002).

Biocides are likely to have multiple target sites within the cell and their mechanisms of action are often multifunctional (Russell, 2002, Walsh et al., 2003a, 2003b). Efflux pumps contribute in a major way to the microbial resistance to antibiotics and biocides (Gilbert & McBain, 2003, Levy, 2002, Poole, 2002). Biofilm ecosystems are known to possess decreased sensitivity to antimicrobials (O'Toole et al., 2000, Donlan & Costerton, 2002, Simoés et al., 2003, Gilbert & McBain, 2003). MIC (Minimal Inhibitory Concentration) values for microbes in the stationary phase or in biofilms have been reported to be orders of magnitude higher than those of actively growing cells (Gilbert & McBain, 2003).

It has been suggested that a general stress response to nutrient depletion and the onset of stationary phase initiates the adaptation of resting or dormant phenotypes in bacteria. These dormant phenotypes are analogous to endospores, which are resistant to numerous physical and chemical agents, including biocides (Gilbert & McBain, 2003). In addition, induction of starvation proteins and alterations in the membrane composition have been reported to increase resistance to environmental stresses (Walsh et al., 2003a, 2003b).

Restriction of growth of harmful microbes in different environments and matrices is difficult. Although on the one hand there is a need to minimize the amount of antimicrobial agents used, e.g. by using the hurdle concept (Leistner, 2000), there also is a threat of increasing antimicrobial resistance when doing so. There are some reports indicating that increased use of bacteriostatic (sublethal), rather that bactericidal (lethal), food preservation systems may contribute to the development and dissemination of antibiotic resistance in food-related pathogens (McMahon et al., 2007). Hence, additional research on microbial adaptation, stress response as well as understanding the mechanisms of action of antimicrobial agents (and factors affecting their mode) both in laboratory and practical applications is needed. Microbial mutant strains isolated or produced under various stress conditions can be useful tools in this research.

Mechanistic studies on the action of antimicrobial chemicals advance our understanding of their potential applications. Most biocides must traverse the outer cell layer(s) of microbes to reach their target sites (Russell, 2002). Therefore, permeabilizers can enhance the activity of biocides and other antimicrobial agents.

2. Aims of the study

The overall aim of the study was to increase knowledge of the mode of action of permeabilizers and to identify new permeabilizers against Gram-negative bacteria.

1. Lactic acid has traditionally been used as a food preservative. We wanted to study whether the preservative activity of lactic acid is related more to the permeabilizing activity than to mere lowering of pH. We thus elucidated whether lactic acid is a permeabilizer, acting synergistically by increasing the activity of other antimicrobial agents.

2. Antimicrobial activity of berry extracts has been widely reported. However, in many cases these extracts are complex mixtures of phenolic compounds and organic acids. We aimed to identify the effect of berry-associated organic acids on the OM of *Salmonella*.

3. The permeabilizing action of EDTA is usually considered to result from LPS release and from consequent perturbations of OM structure and function, but unexplained features in the mechanism still exist. It is not known, for example, why only a certain proportion of LPS is released. We aimed to understand how the classical chelator, EDTA, destablizes the OM of *Salmonella*.

4. In the gastrointestinal tract phenolic berry compounds are metabolized by colonic microbes and converted into various derivatives. We aimed to identify whether colonic metabolites of phenolic compounds are permeabilizers and whether they are able to potentiate the activity of other antimicrobials.

5. Complex microbial biofilms in outdoor environments cause biodeterioration of various materials, including historical monuments. Gram-negative bacteria play an important role in the stabilization of biofilms. We aimed to determine and characterize the effects of selected permeabilizers on the OM of environmental Gram-negative bacteria isolated from biodeteriorated surfaces and to study whether combinations of biocides/permeabilizers can diminish biofilm formation. Especially, we wanted to study the activity of polyethyleneimine on the OM of *Pseudomonas.*

3. Materials and methods

The microbes used in this study and their origin are presented in Table 3. Microbes were obtained from VTT Culture Collection.

Table 3. Target bacteria used in this study, along with their isolation origin.

Strain	Codes	Origin (comment)	Paper
Escherichia coli O157:H7	ATCC 35150 VTT E-97836	human feces	I
Pseudomonas aeruginosa	ATCC 9027 VTT E-96728	ear infection	I
Salmonella enterica sv. Typhimurium	SL696 VTT E-981151	human feces	I, III
Salmonella enterica sv. Typhimurium	ATCC 13311^T VTT E-95582^T	human feces	II
S. enterica sv. Typhimurium	SH5014 VTT E-012041	an *rfaJ* mutant producing rough LPS of chemotype Rb2	III
S. enterica sv. Infantis	VTT E-97738	broiler, Finland	III
Sinorhizobium morelense	VTT E-022105	marble on fresco, Italy	IV
Pseudomonas sp.	VTT E-022106	marble on fresco, Italy	IV
Stenotrophomonas nitritireducens	VTT E-022107	marble on fresco, Italy	IV
Pseudomonas sp.	VTT E-022217	marble on fresco, Italy	IV
Pseudomonas sp.	VTT E-052906	sandstone, Scotland	IV
Pseudomonas sp.	VTT E-052911	sandstone, Scotland	IV

A summary of the methods used in this study is presented in Table 4. Detailed information about the methods is presented in the original publications, Papers I–IV.

Table 4. Methods used in this study.

Assay	Described in paper	References
Permeability		
Uptake of a fluorescent probe, NPN	I, II, III, IV	Loh et al. (1984), Helander & Mattila-Sandholm (2000)
* effect of divalent cations on NPN uptake	I, II, III, IV	
Susceptibility to hydrophobic antibiotics	I, III, IV	
* Agar diffusion method	III, IV	Vaara (1981), Helander et al. (1997a)
* Automated turbidometry	IV	Helander et al. (1997a), Raaska et al. (1999)
Bacteriolysis		
* turbidometric measurement of cell lysis (lysozyme and detergent (SDS and Triton X-100 lysis)	I, III	Helander et al. (1997a)
Release of LPS and other lipid material		
Release of LPS-specific lipids and glycerophospholipids	I, II	Helander et al. (1998a)
* GC-MS (fatty acid analysis)		
LPS release, SDS-PAGE analysis	I, III	Helander et al. (1998a), Hitchcock & Brown, (1983)
Radiolabelling of lipopolysaccharide, release of [^{14}C]galactose labelled-LPS	II, III	Hukari et al. (1986)
Microscopic methods		
Atomic force microscopy	IV	Bolshakova et al. (2004), Meincken et al. (2005)
Growth inhibition		
Automated turbidometry	IV	Raaska et al. (1999)
Microtiter plate assay for biofilm formation	III	Kolari et al. (2003)
Identification of bacteria		
partial 16S rRNA gene sequencing	IV	Saarela et al. (2004)

4. Results and discussion

4.1 Organic acids as permeabilizers

In addition to their ability to reduce the environmental pH, organic acids are reported to be membrane-active agents and hence potential permeabilizers (Doores, 1993). Organic acids have a long tradition of use in the food industry and in the treatment e.g. of animal feed (Ricke, 2003). The effect of lactic acid on the permeability properties of the OM was characterized using *E. coli*, *P. aeruginosa*, *S.* Typhimurium and *S.* Infantis as target strains (Paper I, III). Antimicrobial activity of berry extracts has been widely reported (e.g. Puupponen-Pimiä et al., 2005). However, in many cases these extracts are complex mixtures of phenolic compounds and organic acids. In order to clarify the antimicrobial role of berry-derived organic acids we studied their effect on the OM of *Salmonella* (Paper III). To reveal the effects of acids on the OM permeability uptake of a fluorescent probe (NPN assay), sensitization to lysis caused by detergents and release of LPS specific lipids was monitored.

4.1.1 NPN uptake induced by organic acids

NPN is a hydrophobic probe of which the quantum yield is greatly enhanced in glycerophospholipid as opposed to aqueous environments. Normally NPN is largely excluded by Gram-negative bacteria. Enhanced uptake of NPN occurs in bacterial suspensions containing cells whit a damaged and functionally invalid OM (Loh et al., 1984, Helander & Mattila-Sandholm, 2000). For all studied bacteria, lactic acid brought about a significantly higher NPN uptake than hydrochlorid acid (Paper I, Table 1). The effect was already seen with 5 mM lactic acid (pH 4.0); only with *E. coli* was the NPN uptake further enhanced by the higher concentration of lactic acid (10 mM, pH 3.6). The strongest response was observed in the serovar Typhimurium, but all of the test bacteria responded more strongly to lactic acid than to the classical permeabilizer EDTA. The addition of an equimolar concentration of $MgCl_2$ together with lactic acid decreased the NPN uptake slightly but significantly for *E. coli* and *P. aeruginosa,* whereas in the serovar Typhimurium the effect was insignificant. $MgCl_2$ addition also diminished the effect of HCl, but only in *E. coli*. *P. aeruginosa*

reacted with higher uptake values for HCl in the presence of $MgCl_2$ than in its absence (Paper I, Table 1). The responses to EDTA differed characteristically among the three bacterial species, *P. aeruginosa* reacting most prominently and *E. coli* with the lowest figures; $MgCl_2$ addition abolished the effect in all cases. The above effect was generally similar in the presence of KCN, except in case of *P. aeruginosa*, for which the effect of HCl with KCN was enhanced to a level similar to that obtained with lactic acid bacteria (Paper I, Table 1).

Malic acid, lactic acid and 2-hydroxyhexanoic acid (pH 4.0) brought about a significantly higher NPN uptake in *S.* Typhimurium E-981151, *S.* Infantis E-997738 and *S.* Typhimurium E-012041 (SH5014, Rb2 mutant) compared to HCl (Paper III, Figures 2a–2c). Lactic acid and 2-hydroxyhexanoic acid were more effective than malic acid (Paper III, Figures 2a–2c). The response caused by malic acid (pH 4.0) in the rough mutant *S.* Typhimurium E-012041 cells was lower than in the smooth target strains. Benzoic acid and sorbic acid at pH 4.4 also significantly increased the NPN uptake of the cells. Each strain reacted to malic acid, lactic acid and 2-hydroxyhexanoic acid more strongly than to EDTA (Paper III; Figures 2a–c). $MgCl_2$ addition only slightly decreased the activity of lactic acid, benzoic acid and 2-hydroxyhexanoic acid, whereas NPN uptake-inducing activity of sorbic acid (5 mM) in *S.* Typhimurium E-981151 was abolished by $MgCl_2$ addition.

Disruption of the OM by acids can possibly involve the action of both dissociated and undissociated forms (Doores, 1993). Our findings that hydrochloric acid causes significant OM damage at pH 4.0 shows that the disintegration of the LPS layer can be caused by a fully dissociated acid. The additional OM-disintegrating effect demonstrated for lactic acid, malic acid, 2-hydroxycaproic acid and benzoic acid is propably due to the action of undissociated acid molecules. For example, at pH 4.0 ca. 40% and at pH 3.6 ca. 60% of lactic acid is present in the undissociated form. This conclusion is further supported by our finding that potassium lactate at concentrations up to 10 mM (pH 6.8) had no NPN uptake-enhancing activity on the serovar Typhimurium (data not shown, Paper I). Although the addition of $MgCl_2$ to the NPN assay system together with lactic acid resulted in reduced NPN uptake in *P.aeruginosa* and *E. coli* especially in the presence of KCN, these effects cannot be regarded as indicative of chelation of cations from the OM, since similar effects were observed with HCl and *E. coli* (Paper I). A more likely mechanism than

chelation would be protonation of anionic components such as carboxyl and phosphate groups and the consequent weakening of molecular interactions between OM components. It is plausible that rather than interacting directly with the acid molecule, $MgCl_2$ stabilizes the OM, making it more resistant to acid challenge. As expected, excess Mg^{2+} abolished the NPN uptake induced by EDTA with all the bacterial species studied, indicating that the effect of EDTA was due solely to chelation, as proposed by Leive (1965). The mechanisms of action of malic acid, 2-hydroxyhexanonic acid, benzoic acid and sorbic acid are propably to be similar to that suggested for lactic acid, since similar effects were also observed with $MgCl_2$ for these acids (Paper III).

4.1.2 Effect of acids on bacteriolysis

To further investigate the permeabilizing effect of lactic acid, its effect on the sensitivity of bacteria towards lysozyme and the detergents SDS and Triton X-100 was measured. Similar assays were performed with HCl (pH 3.6) and KCN (1 mM) (Paper I). Lactic acid had a strong sensitizing effect to SDS in each species; similar effect to the nonionic detergent Triton X-100 was also observed, especially in *P. aeruginosa* (Paper I, Table 2). This strain was also strongly sensitized by lactic acid to the lytic action of lysozyme. In enteric bacteria hydrochloric acid brought about significantly weaker sensitizing effects to SDS than lactic acid. However, *P. aeruginosa* was strongly affected; *P. aeruginosa* was also sensitized to Triton X-100 by HCl, but less strongly to lysozyme than by lactic acid. Although KCN had a slight effect on the SDS sensitivity of each species and also a minimal effect with Triton X-100 on *P.aeruginosa*, it was evident that de-energization of the bacterial cells did not have any major impact on their permeability properties.

4.1.3 Acids induce LPS release

Cell-free supernatants of serovar Typhimurium E-981151 after the treatment with lactic acid, HCl, or EDTA were analyzed for SDS-PAGE to investigate the possible release of LPS (Paper 1). Whereas very little LPS was present in the supernatants of untreated cells, the supernants of acid-treated suspensions gave prominent ladder patterns characteristic of serovar Typhimurium smooth-type

LPS (Paper I, Figure 1). Based on visual estimation of the intensity of the staining, the supernatant of lactic acid-treated bacteria contained more LPS than those derived from treatments with HCl or EDTA. *S.* Typhimurium E-981151 supernatants were also subjected to fatty acid analysis to obtain both qualitative and quantative data on the released lipid material. Analysis results confirmed that lactic acid had been the most active acid with respect to LPS release, as indicated by the greatest sum of LPS-specific fatty acids C12:0, C14:0 and C3-OH-14:0 (Paper I, Table 3). In addition to LPS, other lipid material (glycerophosholipids) was also released, as represented by the unsaturated fatty acids detected in the supernatants. However, LPS-specific fatty acids accounted for a greater proportion in the acid supernatants as compared with that of the control, suggesting a preferential release of LPS (Paper I, Table 3).

The effects of lactic acid, malic acid, benzoid acid, sorbic acid, 2-hydroxyhexanoic acid, HCl, or EDTA on the release of LPS in *S.* Infantis E-97738 was studied using radiolabelled LPS (Paper III). EDTA-induced [^{14}C]Gal-LPS release in *S.* Infantis E-97738 was 29±8% (Paper III, Table 1). Compared with the pH 7 treatment, malic acid, lactic acid, benzoic acid, sorbic acid and 2-hydroxyhexanoic acid (all at pH 4.0) induced significant release of [^{14}C]Gal-LPS from *S.* Infantis E-97738. The induced [^{14}C]Gal-LPS releases for the treatments were 39±3%, 33±1%, 32±2%, 33±2% and 34±4%, respectively. However, no significant difference for lactic acid or benzoic acid treatments compared with the hydrochloric acid (pH 4.0) was observed in *S.* Infantis. The amount of malic acid-induced [^{14}C]Gal-LPS release was significantly higher compared with the release induced by hydrochloric acid (pH 4.0).

The results clearly show that lactic acid is a powerful permeabilizer at concentrations of 5–10 mM, the effect being stronger that that of a mineral acid (HCl) at similar pH values (Paper I). This finding is significant as it is in accord with the great preservative potential of lactic acid, and furthermore suggests that the lactic acid present in naturally fermented products may potentiate the antimicrobial action of other compounds against Gram-negative bacteria. The organic acids examined in this study, i.e. malic acid, lactic acid, benzoic acid, sorbic acid and 2-hydroxyhexanoic acid, efficiently destabilized and disintegrated the OM of all target strains (Paper III). Citric acid, another organic acid abundant in berries, has earlier been shown to destabilize and permeabilize Gram-negative bacteria (Helander & Mattila-Sandholm, 2000). Since $MgCl_2$

addition abolished the majority of the OM-disintegrating activity of malic acid, part of the activity may occur by chelation of divalent cations from the OM or intercalation into the OM with the replacement of stabilizing cations.

Our data is in accordance with earlier findings indicating that organic acids, including lactic acid, cause sublethal injury in Gram-negative bacteria, as indicated by their decreased viability on bile salt-containing agar (Roth & Keenan, 1971, Hirshfield et al., 2003). The permeabilizing capacity of organic acids, especially lactic acid, has a number of important consequences. Above all, lactic acid should be able to potentiate the antimicrobial activity of other compounds against Gram-negative bacteria. In fermented low-pH products obtained using lactic acid starter culture bacteria, numerous metabolites are present that are too lipophilic or too large to effectively penetrate the intact Gram-negative bacterial OM (Servin, 2004), but which could potentially do so in the presence of lactic acid. Lactic acid has been identified as one of the major components for antimicrobial activity of some probiotic strains, such as *Lactobacillus rhamnosus* GG (De Keersmaecker et al., 2006). Fayol-Messoudi et al. (2005) suggested that the mechanism of the antimicrobial activity of probiotic *Lactobacillus* strains against *Salmonella* is multifunctional and may be due to the synergistic activity of lactic acid and the secreted non-lactic molecules.

Organic acids, e.g. lactic acid and formic acid, have traditionally been used for the treatment of animal feeds and as food preservatives (Holzapfel et al., 1995, Ross et al., 2002). Recently, Zhao et al. (2006) reported that various combinations of lactic acid, calcium sulphate and sodium benzoate/butyric acid effectively inactivated enterohemorrhagic *E. coli* in rumen content- or faeces-contaminated drinking water for cattle. However, there have been reports indicating that acid adaptation of pathogenic strains can lead to an increased resistance to antimicrobial agents and better survival in acidic conditions (Hirshfield et al., 2003, Bjornsdottir et al., 2006). Therefore, the impact of such adaptive responses on the permeability properties of Gram-negative bacteria should be studied.

4.2 Effect of EDTA on the OM of *Salmonella*

The permeabilizing action of EDTA is usually considered to result from LPS release and from consequent perturbations of the OM structure and function (Leive, 1965, Vaara, 1992), but unexplained features in the mechanism of action still exist. It is not known, for instance, why only a certain proportion of LPS is released. It was shown by Hukari et al. (1986) that the macromolecular quality (LPS chain length distribution) of EDTA-released LPS vs. cell-bound LPS was identical. It can, however, be postulated that the releasable fraction differs from the non-releasable one in some structural aspect that is related to the stabilizing effect of divalent cations, possibly in the level of charged groups in the core oligosaccaharide and lipid A. By using *S.* Typhimurium E-95582^{T} as a target microbe we wanted to study the effects induced by EDTA treatment in cells grown to different growth phases and under various ion concentrations (Paper II).

4.2.1 NPN uptake by cells in different growth phases

S. enterica sv. Typhimurium E-95582^{T} grown in LB showed a typical growth curve with lag- and exponential phases. The effect of the growth phase on EDTA sensitivity was studied by using cells harvested in different growth phases: early-logarithmic phase (A_{630}= 0.20±0.02), mid-logarithmic phase (A_{630}= 0.50±0.02) and late-logarithmic phase (A_{630}= 0.70±0.02). With 2 mM $CaCl_2$ or $MgCl_2$ supplementation no significant difference in the shape of the growth curve was observed (Paper II).

The NPN uptake of Typhimurium cells grown in LB did not differ significantly in different growth phases (fluorescence levels of control cells from 100 to 200 units, Paper II, Figure 1). However, after treatment with EDTA the cells exhibited increased NPN uptake, although this phenomenon was significant only in the early logarithmic phase of growth. Addition of 2 mM $CaCl_2$ into growth media stabilized the OM, as indicated by the lower NPN uptake of the control cells (Figure 1b, fluorescence levels of less than 100 units). In general, supplementation of cells with Ca^{2+} during growth considerably increased their NPN uptake induced by EDTA, especially in the mid- and late logarithmic phase. Accordingly, significant increases in NPN uptake caused by 1.0 mM EDTA were observed in all growth phases, the earlier phases again exhibiting

higher uptakes. 0.1 mM EDTA was obviously not sufficient in destabilizing and increasing the permeability of the OM in early-logarithmic cells ($A_{630} = 0.2$) grown in LB supplemented with 2 mM $CaCl_2$, indicating the presence of massive ionic interactions within the OM (Figure 1b). In the presence of 2 mM Mg^{2+} the NPN uptake values with 1 mM EDTA (Figure 1c) were alike but not as high as with Ca^{2+} addition. However, the presence of 2 mM Mg^{2+} during cultivation was sufficient in destabilizing and increasing the permeability of the target cells by 0.1 mM EDTA already in the early growth phase. Addition of 1 mM $MgCl_2$ into buffer used in the NPN assay abolished the permeabilizing activity of EDTA (data not shown) and the NPN uptake of target cells was at the same level as in the corresponding control cells. The conclusion from these experiments was that the growth phase has a profound effect on the bacterium's sensitivity to EDTA as assayed by NPN uptake, with early logarithmic phase cells exhibiting particularly high sensitivity. Finch and Brown (1975) observed increased sensitivity to EDTA (cell lysis) in *P. aeruginosa* cells with low growth rates when cells were grown either under carbon limitation or in Ca-enriched medium. Furthermore, increased resistance to EDTA in *P. aeruginosa* was reported for cells in Mg-limited medium. According to Finch and Brown (1975) the removal of cations from the cell membrane is due to the greater affinity of the cations for EDTA than for cell membrane components. In addition, the higher stability constant for EDTA interaction with calcium than with magnesium (10.7 and 8.7, respectively) also influences the activity of EDTA in the growth medium.

4.2.2 Sensitization of bacteria to bacteriolysis induced by detergents or lysozyme

Increased permeability of the OM is also manifested as an increased susceptibility to the bacteriolytic action of detergents and to the cell wall-degrading action of lysozyme (Vaara, 1992). To further investigate the sensitivity of Typhimurium cells in different growth phases we therefore tested the effect of EDTA on the susceptibility of Typhimurium to lysozyme- and detergent-induced cell lysis (Paper II). Table 1a showed that significant lysis by Triton X-100 was not observed in control cells, whereas SDS (anionic detergent probe) itself somewhat lysed the control cells. However, treatment with EDTA sensitized LB-grown Typhimurium cells to SDS (0.05 and 0.1%). This sensitisation was quantitatively similar in each growth phase. Early logarithmic

cells grown in LB supplemented with Ca^{2+} and pretreated with 0.1 mM EDTA were more resistant to lysis by 0.05% SDS (Table 1b) than cells grown without Ca^{2+} (Table 1a). These cells were equally sensitised to the action of 0.1% SDS/1 mM EDTA in early and late logarithmic phase. Notably, the presence of 2 mM Ca^{2+} during cultivation rendered the early logarithmic cells sensitive to lysis by 1% Triton X-100/1 mM EDTA. In conclusion, results of the cell lysis experiments did not indicate major differences in the functional properties of Typhimurium OM as function of the growth phase (Paper II).

4.2.3 Release of LPS

Since EDTA appeared to weaken the OM strongly in the early logarithmic phase and since it is known to destabilize OM by liberating LPS, we studied the amount of LPS and lipid material released by EDTA at different growth phases (Paper II). Table 2 shows that EDTA liberated lipid material, including LPS, as indicated by the LPS-specific fatty acids C12:0, C14:0, 3-OH-14:0, and glycerophospholipid, as indicated by fatty acids C16:0, C16:1, C18:1. Ca^{2+} supplementation during growth increased the total amount of liberated fatty acids. However, no significant differences were detected in the amounts of LPS liberated by EDTA from cells grown to the early or late logarithmic phase. A similar result was obtained from experiments involving specific labelling of LPS ([^{14}C]Gal-LPS) and analysis of EDTA-releasable [^{14}C]Gal-LPS in three different growth phases (Table 3). The conclusion thus is that the amount of LPS release by EDTA is independent of the growth phase in Typhimurium E-95582^{T}. In Paper III we used radiolabelling of LPS to monitor release of LPS from *S*. Infantis E-97738. EDTA-induced [^{14}C]Galactose-LPS release in *S*. Infantis E-97738 was 29±8% (Paper III, Table 1). This is in accordance with earlier reports, according to which Tris-EDTA releases approximately 30 to 50% of the LPS of smooth *E. coli* and *S*. Typhimurium (Leive, 1974).

The results demonstrate that the effect of EDTA on *S. enterica* cells involves a component that is independent of LPS release, the classical explanation for the mechanism of EDTA-induced permeabilization of Gram-negative bacterial OM. This component was indicated by the significantly higher NPN uptake observed in early logarithmic phase cells compared with late logarithmic phase cells. Release of LPS, measured either by monitoring the amount of LPS-specific fatty acids in cell-free supernatants or radiolabelled LPS in a standardized cell density

of the treated cells, remained virtually unchanged along the growth curve. Another method to test permeabilization, i.e. sensitisation to lytic agents, yielded mostly results paralleling those of the LPS release measurements. It can thus be concluded that in addition to its LPS-releasing mechanism EDTA in the early logarithmic phase of growth acts on cells by another mechanism that does not involve LPS release.

The mechanism underlying the early logarithmic sensitisation to EDTA remains unknown at present, but opens up possibilities concerning stability-affecting properties of the OM components. Since NPN fluorescence is associated with the presence of this hydrophobic probe in a glycerophospholipid environment (Träuble & Overath, 1973), it is evident that in the presence of EDTA early logarithmic cells very easily allow access of NPN to glycerophospholipids either directly on the OM surface or via the periplasm. In the former case the explanation should be found among the interactions between OM components, i.e. there should be demonstrable differences in the structure of OM components as a function of the growth phase. Such differences could be expected to be found in the fine structures of LPS, especially in the degree of substitution of phosphate groups of lipid A and the core oligosaccharide. In addition to EDTA-releasable fraction, there may be a population of LPS of wich interactions with neighbouring components are disturbed by EDTA to an extent that does not, however, result in LPS release. In order to prove this, the LPS obtained from distinct timepoints of the growth phase should be scrutinized for phosphate substituents known to be critical for OM integrity (Helander et al., 1997b, Raetz & Whitfield, 2002, Yethon & Whitfield, 2001b). In addition, LPS fractions which are releasable and non-releasable by EDTA should be studied for similar fine structures, e.g. by using ^{31}P-NMR. In this context it is interesting to note that the report of Kanipest et al. (2001) demonstrated in *E. coli* that the critical parameter determining the presence or absence of phosphoethanolamine was the $CaCl_2$ concentration in the medium. They observed a novel $CaCl_2$-induced enzyme that modifies the outer Kdo moiety of *E. coli* LPS with a phosphoethanolamine group in the presence of 5–50 mM $CaCl_2$. Such a modification would increase the average resistance of LPS molecules towards the releasing action of EDTA, as molecules capped with phosphoethanolamine in Kdo are less prone to be stabilized with divalent cations. Our finding that the addition of Ca^{2+} ions to the growth medium in some cases stabilized the OM, is in agreement with this finding.

The studies of Kotra et al. (2000) employing atomic force microscopy demonstrated that the OM surface in *E. coli* is not uniform; i.e. LPS molecules form distinct patches with depressions in between. Furthermore, a non-uniform distribution of metal ions in the OM was implied, giving rise to local variations in the interactions between OM components. Our findings are in agreement with this view. Another mechanism could be that efflux pumps that remove substances such as NPN from the periplasm are functionally impaired by EDTA, and that they are present in larger numbers in the early logarithmic phase of growth compared to the late logarithmic phase. In this case the phenomenon is not one of permeabilization but rather a more indirect one. Finally, our results demonstrate that NPN uptake assays should be carried out with cells that have been cultivated in standardized conditions, especially with respect to their growth phase and growth media.

4.3 Effect of colonic microbial metabolites of phenolic compounds on OM of *Salmonella*

Members of the genus *Salmonella* cause gastroenteritis and are able to colonize mucus of the gastrointestinal tract (Humphrey, 2004). Many of the virulence factors and adhesion functions are related to the surface structures of *Salmonella* (Trent et al., 2006). Hence we wanted to study the effect of colonic microbial metabolites of berry-derived phenolic compounds on the permeability of *Salmonella*.

4.3.1 Effect of tested samples on the uptake of NPN

To reveal changes in OM permeability, two smooth and one rough type (LPS chemotype Rb2) *S. enterica* were selected for NPN uptake studies. The detailed results of the NPN uptake experiments with the treatments, including the effect of addition of $MgCl_2$ in the assay buffer, are shown in Figures 1a–c (Paper III). EDTA caused significant NPN uptake in the smooth *S.* Typhimurium E-981151 and *S.* Infantis E-97738 cells at the concentrations of 0.1 and 1.0 mM.

3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3-(3,4-dihydroxyphenyl)propionic acid (3,4-diHPP) and 3-(4-hydroxyphenyl)propionic

acid (pHPP) destabilized and disintegrated all of the target bacteria and brought about a significantly higher NPN uptake than hydrochloric acid. The effect was already seen at 2.5 mM (pH 5.0) concentration of the tested metabolites. 3-phenylpropionic acid and 3-HPP also destabilised the cells and significantly increased their NPN uptake (Paper III; Figures 2a–2c).

Divalent cations are known to inhibit the action of many outer membrane permeabilizers which act by chelation or replacement of divalent cations in the OM. We therefore tested whether Mg^{2+} could affect the activity of the tested agents. Figures 2a–2b show that for the smooth strains 1 mM $MgCl_2$ addition almost totally abolished the activity of EDTA. In addition, $MgCl_2$ addition diminished the permeabilizing activity of 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-diHPP and pHPP at the concentration of 2.5 mM for all target strains. With higher (10 mM) test agent concentrations 1 mM $MgCl_2$ addition only slightly decreased the NPN uptake of the cells.

4.3.2 Antibiotic susceptibility

A sensitizing effect to hydrophobic antibiotics is one of the indications of OM-permeabilizing action. We tested the susceptibility of the target strains to a set of hydrophobic antibiotics (clindamycin, fucidin and novobiocin) by the agar diffusion method on plates containing microbial metabolites of phenolic compounds. 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid and 3,4-diHPP sensitized *S.* Typhimurium E-981151 cells to novobiocin. *S.* Infantis E-97738 cells were sensitized only by 3-hydroxyphenylacetic acid to novobiocin. The rough *S.* Typhimurium E-012041 cells were sensitized to novobiocin by 3,4-dihydroxyphenylacetic acid and 3,4-diHPP. Susceptibility of target strains to the other tested antibiotics was not enhanced.

4.3.3 Release of LPS and cell lysis

Table 1 summarizes the results of specific labelling of [^{14}C]galactose-LPS and LPS release (Paper III). EDTA-induced [^{14}C]Gal-LPS release in *S.* Infantis E-97738 was 29±8%. Treatment with 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-diHPP or pHPP (2.5 mM, pH 5.0) induced 10–13% release of [^{14}C]Gal-LPS from *S.* Infantis E-97738, which was at same level (10±4%) as in the treatment with hydrochloric acid (pH 5.0). Based on the visual

estimation of the intensity of the silver-stained SDS-PAGE gel, LPS release for *S*. Infantis caused by 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-diHPP and pHPP was evident. In fact, with *S*. Typhimurium E-981151 visual inspection of the SDS-PAGE gels revealed higher amounts of LPS in the treatment supernatants compared with *S*. Infantis. No sensitization to lysozyme was observed with the microbial metabolites at 2.5 mM concentration. The permeation of lysozyme apparently requires extensive disorganization of the OM, such as that resulting from massive loss of LPS induced by EDTA (reviewed in Vaara, 1992).

The results obtained in the NPN uptake assay demonstrate that 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3-phenylpropionic acid, 3,4-dihydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid and 3-hydroxyphenylpropionic acid efficiently destabilized the OM of *Salmonella* as indicated by an increase in the NPN uptake. The OM-destabilizing activity of the compounds was partially abolished by $MgCl_2$ addition, indicating that part of their activity is based on the removal of OM-stabilizing divalent cations (Paper III). *S*. Infantis E-97738 was less sensitive than *S*. Typhimurium E-981151 cells, a phenomenon which is possibly related to differences in their LPS structures. Since $MgCl_2$ addition abolished the majority of the OM-disintegrating activity of 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-diHPP and pHPP at 2.5 mM concentration, part of their activity may be due to chelation of divalent cations from the OM or intercalation into the OM with the replacement of stabilizing cations. However, since the *S*. Typhimurium E-012041 (SH5014) cells, which produce rough LPS of chemotype Rb2 and lack the O-specific chain and most of the outer core oligosaccharides in their LPS, were also destabilized by these compounds, our results indicate that the outer core and the O-specific chain played no significant role in the effects caused by these substances. This is in accordance with the general conclusion that charged regions in the lipid A and inner core oligosaccharide are chiefly responsible for maintenance of the barrier function in the OM (Vaara, 1992, Nikaido, 2003).

3,4-dihydroxyphenylacetic acid and 3,4-diHPP sensitized *S*. Typhimurium cells to novobiocin, whereas only 3-hydroxyphenylacetic acid sensitized *S*. Infantis to novobiocin. This suggests that dihydroxy acids can disintegrate the OM of *S*. Typhimurium more strongly than acids with only one hydroxyl group. However, with *S*. Infantis this phenomenon was not observed either in the

antibiotic sensitization or in the LPS release assay. We showed in Paper I that lactic acid (another hydroxy acid) acts as a permeabilizer of Gram-negative bacteria. Veldhuizen et al. (2006) recently reported that the hydroxyl group of carvacrol affects its antimicrobial mode of action.

According to Scalbert and Williamson (2000) the local concentrations of colonic microbial metabolites in the colon are around 3 mM. Our data (OM destabilizing activity using a 2.5 mM test concentration) suggests that microbial colonic metabolites of phenolic compounds may play a role in the defense of GI tract against gastro-intestinal pathogens. However, the physiological importance of this phenomenon requires further investigation. In addition, conditions in the lumen can affect the inhibitory activity of the compounds. The mild acidity conditions of the large intestine (pH 5.4, Gee et al., 1999) can be favourable for the permeabilizing action of phenolic metabolites. In addition, there may be microenvironments where the accumulation of colonic metabolites are high enough to destabilise of the OM and potentiate the activity of other antimicrobial agents.

4.4 Weakening of environmental Gram-negative isolates with permabilizers

4.4.1 Permeability assays

Environmental isolates were selected for NPN uptake studies in order to reveal possible changes in the OM permeability of Gram-negative bacteria causing biodeterioration (Paper IV). NPN fluorescence is associated with the presence of this hydrophobic probe in a glycerophospholipid environment (Loh et al., 1984), and increased fluorescence values indicate weakening of the OM. The detailed results of the NPN uptake experiments with EDTA, PEI, DMSA, nitrilotriacetic acid (NTA), sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) and benzalkonium chloride (BC) are presented in Table 2 (Paper IV). EDTA (1 mM), PEI (10 µg ml^{-1}) and DMSA (1 mM) brought about a significantly higher NPN uptake than control treatments with all other strains except *S. morelense* E2105 (Paper IV, Table 2). BC (0.001% w/v) weakened the outer membrane of the tested microbes as indicated by a significant increase in the NPN uptake. Addition of 1 mM $MgCl_2$ into the buffer used in the NPN assay abolished the permeabilizing

activities of 0.1 mM EDTA and diminished the permeabilizing activity of PEI. The OM destabilizing activity of 1 mM EDTA and DMSA was only partially abolished by $MgCl_2$. 1 mM DMSA supplementation resulted in pH 4.5 in the test assay, whereas for the other treatments the pH remained at pH 7.0. NTA at 1 mM concentrations did not significantly increase the NPN uptake of the target strains. AOT (1 mM) increased the NPN uptake of *Pseudomonas* sp. E2906 strain, whereas NPN uptake of other microbes was only slightly affected. Bansal-Mutalik and Gaikar (2003) reported that AOT was capable of permeabilizing *E. coli* cells and causing selective enzyme (penicillin acylase) release. In our study AOT (1 mM) increased the NPN uptake of *Pseudomonas* sp. E2906 strain, whereas NPN uptake of other microbes was only slightly affected. NTA has been reported to increase the sensitivity of Gram-negative bacteria to hydrophobic antibiotics (Ayres et al., 1998, Vaara, 1992). In our study 1 mM NTA weakly destabilized *Pseudomonas* sp. E2106 and *St. nitritireducens* E2107 cells. However, NTA has been classified as possibly carcinogenic (Anonymous, 1999) and therefore it is not suitable to be used in biocide formulations intended for environmental applications, although it might have other application areas.

Succimer (DMSA) has been reported to be a potential remover of smear layers in dental applications (Vineeta et al., 2001). In our study succimer was capable of destabilizing the OM of all tested strains. In the NPN uptake assay $MgCl_2$ addition only slightly decreased the OM disintegrating activity of DMSA, indicating that this activity was only partially related to the removal of stabilizing divalent cations from the OM. Succimer is a hydroxy acid compound and thereby part of the permeabilizing activity is related to the acidity and structure of the compound. Lactic acid, another hydroxy acid, has been shown to be a potent permeabilizer (Paper I).

PEI is a weakly basic aliphatic polymer which is polycationic due to the presence of primary, secondary and tertiary amino groups (Bahulekar et al., 1991). Helander et al. (1997a) demonstrated that PEI is a potent permeabilizer of the OM of pathogenic Gram-negative bacteria, as it sensitized *E. coli*, *P. aeruginosa* and *S.* Typhimurium to hydrophobic antibiotics and detergents. Helander and coworkers (1998b) also demonstrated that PEI intercalated in the OM and increased the membrane surface area without liberation of LPS-associated cell material from *Salmonella*. Our study confirms that PEI is also capable of

permeabilizing Gram-negative environmental strains, representing *Pseudomonas* and *Stenotrophomonas* species, since significant NPN uptake and increased sensitivity to hydrophobic antibiotics was observed with these strains (Paper IV).

4.4.2 Antibiotic suspectibility

A sensitizing effect to hydrophobic antibiotics is one of the indications of OM-permeabilizing action. We tested the susceptibility of the target strains to a set of hydrophobic antibiotics (clindamycin, rifampicin, novobiocin, erythromycin and fucidin) by the agar diffusion method on plates containing different concentrations of PEI. PEI induced an increased susceptibility of *Pseudomonas* sp. E2106 to erythromycin, novobiocin and fusidin (Paper IV, Table 3). However, the susceptibility of *St. nitritireducens* E2107 to these antibiotics was not significantly enhanced by PEI addition in agar plate tests (Paper IV, Table 3). PEI supplementation slightly increased the susceptibility of target strains to rifampicin. Susceptibility of *S. morelense* E2105 to novobiocin was only slightly enhanced by PEI supplementation. The effect of PEI on the growth of *Pseudomonas* sp. E2106 and *St. nitritireducens* E2107 was also tested using an automated turbidometer, Bioscreen (Paper IV, Figures 1a–b). In the agar diffusion test a higher concentration of PEI was required (>25 μg ml^{-1}) than in the suspensions (10 μg ml^{-1}) for the sensitization of *Pseudomonas* sp. E2106 cells to novobiocin (Figure 1a). In suspension experiments supplementation by 10 μg of PEI ml^{-1} enhanced the susceptibility of *Pseudomonas* sp. E2106 and *St. nitritireducens* E2107 cells to novobiocin. However, the growth of *St. nitritireducens* E2107 was not fully prevented by the combination of PEI and novobiocin even at higher concentrations tested (Figure 1b).

Intrinsic and acquired multidrug resistance in Gram-negative bacteria is related to the synergy between limited OM permeability and energy-dependent multidrug efflux pumps (Poole, 2002). *S. morelense* is an opportunistic pathogen and has been reported to be highly resistant to several antibiotics (Wang et al., 2002). Our environmental *S. morelense* isolate, E2105, was sensitive to clindamycin, rifampicin, novobiocin, erythoromycin and fucidin. Addition of PEI slightly increased the susceptibility of this strain to novobiocin. However, *S. morelense* E2105 seemed to have a weak OM structure since in the NPN uptake assay the uptake values were high already in control cells and no statistically significant difference between various treatments was observed. This weak structure was

probably related to the number of stabilizing divalent cations in the OM, since $MgCl_2$ addition stabilized the control cells but the permeabilizing activity of EDTA was not completely abolished by the $MgCl_2$ addition.

4.4.3 Prevention of biofilm formation on PVP plates and enhancement of *in vitro* antimicrobial activity

We applied the biofilm formation assay developed by Kolari et al. (2003) to study the biofilm formation by six environmental isolates and the ability of permeabilizers and BC to prevent biofilm formation. The most effective biofilm formers were *St. nitritireducens* E2107 and *Pseudomonas* sp. strains E2106, E2906 and E2911 (Paper IV, Figure 4). EDTA at 1 mM concentration prevented biofilm formation of the tested strains. BC at a concentration of 0.01% significantly prevented biofilm formation of all tested strains compared with the control treatment. Even a lower BC concentration diminished the biofilm formation of the strains compared with the control treatments, *Pseudomonas* sp. E2106 being less affected than the other strains. DMSA at a concentration of 1 mM prevented biofilm formation by *S. morelense* E2105 and *Pseudomonas* sp. E2906 and E2911. Supplementation by PEI (10 μg ml^{-1}) did not significantly decrease the biofilm formation compared to the control. To further study the capability of selected permeabilizers to increase the efficacy of BC in suspensions, an automated turbidometric assay was used to monitor growth of target strains by following optical density of the samples. BC alone had a minor growth inhibitory activity against *Pseudomonas* sp. E2106 and E2217 strains (Paper IV, Table 4). Supplementation with PEI (10 μg ml^{-1}) significantly increased the activity of BC towards the tested Pseudomonas strains, whereas EDTA (0.1 mM) supplementation did not increase the activity. In the test assay supplementation by PEI alone diminished the growth of *Pseudomonas* sp. E2106.

Pseudomonas species are able to degrade chloride compounds and are not very sensitive to quaternary ammonium compounds (Gilbert & McBain, 2003). Loughlin et al. (2002) reported that *P. aeruginosa* cells generated stable resistance to BC during passage in sub-minimal inhibitory concentrations of BC, and this resistance was also later retained in the absence of the disinfectant. In addition, a cross-resistance to the membrane-active antibiotic polymyxin B was also detected. In our studies in the biofilm formation assay *Pseudomonas* sp.

E2106 was the most resistant strain to BC among the tested strains. EDTA and PEI enhanced the activity of BC towards *Pseudomonas* in suspension experiments. In addition, our studies showed that BC disintegrated the OM of the target cells, as indicated by an increased NPN uptake. Recently it was reported that EDTA at high (50 mM) concentration caused a rapid dispersion of *P. aeruginosa* cells from biofilms by chelation of several divalent cations that are required to stabilize the biofilm matrix (Banin et al., 2006).

4.4.4 Atomic force microscopy (AFM) studies

In order to visualize the effects caused by EDTA and PEI on *Pseudomonas* sp. E2106 we applied AFM for the imaging of treated cells (Paper IV). Topographic images of the control *Pseudomonas* sp. E2106 cells revealed a compact and smooth surface without notable ruptures or pores on the cell surface (Paper IV, Figure 2a). Phase contrast images of the control cells revealed that the hydrophilic surface was uniform. Magnification of the topographic images also revealed a uniform OM structure (Figure 3a). The surfaces of the 1 mM EDTA treated cells visualized in topographic images were rough and the outer membrane surface appeared damaged, indicating release of LPS and weakening of OM structure (Paper IV, Figure 2b). Phase contrast images revealed large areas with different hydrophilicity/hydrophobicity on the cell surface. The magnification of the topographic images showed extensive disruption of the LPS layer (Figure 3b). The release of LPS from the surface of EDTA-treated cells resulted in large and irregularly shaped pits where the cytoplasmic membrane was revealed. The effect of PEI (Paper IV, Figure 2c) was different from that of EDTA. Treatment of the cells with PEI flocculated the *Pseudomonas* cells, causing aggregation and adhesion. In addition, the cells were swollen, with increased cell surface area and bulges. Magnification of the topographic image showed smooth OM surface with bulges and an increased surface roughness as compared with the control cells (Figure 3c). Permeablizers affected the average surface roughness (Rms(Rq)) of the cells. EDTA- and PEI-treated cells had significantly higher surface roughness compared to the control cells, 3.23±0.49, 7.48±1.46 and 2.06±0.45, respectively (Paper IV). In this study the cells were air-dried on mica surface. However, in further trials immobilization of cells to gelatine-coated mica surface (Doktycz et al., 2003) would allow imaging of cells in liquid, thus resulting in more intact cell surface.

The massive changes on the OM of *Pseudomonas* sp. E2106 due to PEI treatment displayed by AFM images are not surprising, as *Pseudomonas* lipopolysaccharides are typically rich in phosphate groups (Walsh et al., 2000) and *Pseudomonas* cell surface is thus expected to bind polycationic PEI in large amounts. In PEI-treated cells AFM images visualized the capability of PEI to intercalate in the OM and increase the membrane surface area. This observation is in agreement with the results of Helander et al. (1998a), who reported the same phenomenon in *Salmonella* by using transmission electron microscopy. Kotra et al. (2000) studied the effect of EDTA on *E. coli* with AFM and they reported that release of the LPS from the surface resulted in large and irregularly shaped pits where the peptidoglycan layer was exposed. Our AFM images from EDTA-treated *Pseudomonas* cells also revealed patchiness of the damaged OM structure. This non-uniform alteration of the OM by EDTA as revealed by AFM is in accordance with the classical finding that only a certain proportion of LPS can be released by EDTA, indicating the presence of structurally and electrostatically different subpopulations of LPS in the OM (Leive, 1965, 1974, Vaara, 1992). The existence of such structurally distinct LPS populations in spatially separate areas of the OM, as discussed in more detail in Paper I, is further supported by our present findings with AFM.

4.5 Future aspects

Table 5 summarizes the effects of various permeabilizers examined in this study on selected target microbes. Combination of the results obtained from various permeability assays (e.g. uptake of a hydrophobic probe, sensitization to hydrophobic antibiotics and detergents, release of LPS and LPS-specific fatty acids) and AFM image results increases our knowledge of the action of permeabilizers.

Alternative and novel biocide formulations are needed to restrict the growth of harmful microbes in sites were traditional biocides are ineffective. The application of an effective biocide / permeabilizer combination could aid in the destruction of microbial biofilms while allowing the use of reduced concentrations of the biocide. In order to promote enhancement of the activity of biocides, knowledge of the mechanism of permeabilizers and factors influencing their activity is essential.

In environmental applications the effective biocide / permeabilizer combination could aid in the destruction of the microbial biofilms that cause the degradation while allowing the use of reduced concentrations of the biocide. However, the efficacy of the formulated biocide products must be further evaluated on e.g. stone materials with complex microbial communities and in field trials under various environmental conditions. In addition, compatibility of the formulated products with commercial restoration products such as water repellents and consolidation agents must be ensured.

In food applications the efficacy of the formulated products must be further evaluated in various matrices with complex microbial communities. Especially in food applications before antimicrobials/ permeabilizers are applied, the compounds should be reviewed for the toxicological data and possible approval for use in foods. In addition, the effects of the compounds on the organoleptic quality of the foods must also be assessed.

Table 5. Effects of various permeabilizers on selected target microbes.

Target strain/ Test agent	Measured response NPN uptake increase	 Sensitization to hydrophobic and lytic agents	 LPS release
Salmonella enterica sv. Typhimurium E-981151			
Lactic acid	+++	+++	+++
Malic acid	+++	nd	+++
Benzoic acid	+++	nd	++
EDTA	+++	+++	+++
3,4-dihydroxyphenylacetic acid	++	++	+
3-hydroxyphenylacetic acid	++	++	+
3-(3,4-dihydroxypheynyl) propionic acid	++	++	+
Salmonella enterica sv. Infantis E-97738			
Lactic acid	+++	nd	+++
Malic acid	+++	+++	+++
Benzoic acid	+++	nd	+++
Sorbic acid	++	nd	+++
EDTA	+++	+++	+++
3,4-dihydroxyphenylacetic acid	++	-	+
3-hydroxyphenylacetic acid	++	++	+
3-(3,4-dihydroxypheynyl) propionic acid	++	-	+
3-(4-hydroxyphenyl)propionic acid	++	-	+
3-phenylpropionic acid	++	nd	+
3-(3-hydroxyphenyl)propionic acid	++	nd	+
2-hydroxyhexanoic acid	+++	nd	+++
Escherichia coli E-97836			
Lactic acid	+++	+++	+++
EDTA	+++	+++	+++
Pseudomonas aeruginosa E-96728			
Lactic acid	+++	+++	+++
EDTA	+++	+++	+++
Pseudomonas sp. E-022106			
EDTA	+++	+++	+++
PEI	+++	+++	-
Succimer (DMSA)	+++	+++	nd

+++ = strong effect, ++ = medium effect, + = moderate effect, - = no effect, nd = no data

5. Conclusions

The following main conclusions can be drawn from the results obtained during this work.

1. The results show that lactic acid is a powerful permeabilizer at concentrations of 5–10 mM, the effect being significantly stronger than that of a mineral acid (HCl) at similar pH values (Paper I). This finding is in accordance with the great preservative potential of lactic acid and furthermore suggests that lactic acid present in naturally fermented products may potentate the antimicrobial action of other compounds against gram-negative bacteria.

2. Organic acids present in berries, such as malic acid, sorbic acid and benzoic acid, were shown to be efficient permeabilizers of *Salmonella* as shown by increase in the NPN uptake assay and by LPS release (Paper III).

3. EDTA affected the OM much more strongly in the early logarithmic growth phase than in the mid- or late growth exponential phase, as indicated by NPN uptake assay and detergent susceptibility. However, this effect was not paralleled by LPS relase, which remained unchanged as a function of the growth curve. The results indicate that in the early exponential phase the effect of EDTA in *S. enterica* sv. Typhimurium involves a component that is independent of LPS release (Paper II). Further studies should be performed to demonstrate possible differences in inner core phosphate residues by using appropriate NMR techniques. In addition, our results demonstrate that NPN uptake assays should be carried out with cells that have been grown in standardized conditions, especially with respect to their growth phase and growth media.

4. The results obtained in the NPN uptake assay demonstrated that 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acid, 3-(4-hydroxyphenyl)propionic acid, 3-phenylpropionic acid and 3-(3-hydroxyphenyl)propionic acid efficiently destabilized the OM of *Salmonella* as indicated by increase in the uptake of NPN. The OM-destabilizing activity of the compounds

was partially abolished by $MgCl_2$ addition, indicating that part of their activity is based on the removal of OM-stabilizing divalent cations (Paper III).

5. Among the potential permeabilizers examined in this study EDTA, PEI and succimer (DMSA) were shown to be efficient permeabilizers for members of the *Pseudomonas* and *Stenotrophomonas*, as indicated by the increase in the uptake of hydrophopic probe (NPN, Paper IV). AFM is a powerful tool for visualizing OM damage caused by permeabilizers.

Acknowledgements

Studies presented in this thesis were carried out at VTT Technical Research Centre of Finland during the years 1997–2006. The financial support of the Academy of Finland, Tekes – Finnish Funding Agency for Technology and Innovation, The European Commission and VTT is acknowledged.

I thank Professor Juha Ahvenainen for providing excellent working facilities. In addition, I thank Technology Manager Dr. Anu Kaukovirta-Norja for providing me the possibility for writing leave in order to complete this thesis, and for her encourgament during the writing period.

I thank the pre-examiners of the thesis, Professor Airi Palva and Professor Mikael Skurnik for their amendments and comments concerning the manuscript. My custos, Professor Mirja Salkinoja-Salonen, I would like warmly thank for her encouragement and advice during the finalizing of the thesis.

I am deeply indepted to my principal supervisor Docent Ilkka Helander. Even though you had withdrawn from the daily science environment you still had time for discussions and advice when needed. Thank you, Ilkka, for your encouragement during finalising practical work and during the writing of this thesis. Your vast experience and enthusiasm as well as your advice and comments have been priceless. I also thank you for your blues music; although blue, it has given us much joy of life. My second supervisor, Chief Research Scientist, Docent Maria Saarela, I thank for your encouragement and supervision during the last years when I was working in your team.

I thank my co-authors, MSc Eija Skyttä, MSc Kyösti Latva-Kala, Dr. Tiina Mattila-Sandholm (currently at Valio Ldt), Dr. Maija-Liisa Suihko, Dr. Riitta Puupponen-Pimiä, Dr. Kirsi-Marja Oksman-Caldentey and Dr. Liisa Nohynek for pleasent cooperation during the projects and while preparing the manuscripts. In addition, I thank my co-author Dr. Anna-Marja Aura for fruitful discussions while preparing the fourth manuscript. My co-author Dr. Arja Paananen I thank for introducing me to the fascinating world of atomic force microscopy.

I thank Päivi Lepistö, Taina Holm, Tarja Nordenstedt, Helena Hakuli, Anne Heikkinen, Anna-Liisa Ruskeepää and Ulla Vornamo for skillful technical assistance. Likewise I thank all my colleagues at VTT Biotechnology for a creative environment and especially members of the "Probiotic Team" and "Brewing Microbiology Team". Johanna, Maisa, Niina, Anne, Helena, Maija-Liisa, Jaana, Maria, Arja and all others, your optimistic and cheerfull spirits have made the working days rewarding. Dr. Jaana Mättö I thank for fruitful discussions and encourgament while you were working at VTT. Special thanks also to the girls in the third floor (Eija, Satu, Hanna, Kaarina, Outi and Kirsi) for sharing the troubles and joys of everyday life.

My parents, Leena and Jouni Alakomi, I thank you for your care, your support and encourgament during the final steps of this thesis and throughout my entire life. I also thank my sister, Katri, and my brother, Kalle, for your support. The hours spent playing with your lovely children have given much joy of life. I thank my friends, especially Anneli and Outi, for your friendship. Without you and the hours spent together, in the woods picking up berries or in the fields growing up garlic, life would have been more dull. Finally, I would like to express my warmest thanks to Veli for your support and for not loosing your nerves (at least very often) when papers were colonizing our living room in the last stages of finalizing this thesis.

Helsinki, Tapaninkylä April 2007

Hanna-Leena Alakomi

References

Alexander, C. & Rietschel, E.T. (2001). Bacterial lipopolysaccharides and innate immunity. *Journal of Endotoxin Research*. 7, 167–202.

al-Saigh, H., Zweifel, C., Blanco, J., Blanco, J.e., Blanco, M., Usera, M.A. & Stephan, R. (2004). Fecal shedding of *Escherichia coli* O157, *Salmonella*, and *Campylobacter* in Swiss cattle at slaughter. *Journal of Food Protection*. 4, 679–684.

Anonymous. (1999). International Agency for Research on Cancer (IARC). Vol. 63. 338 p. Some chemicals that cause tumors of the kidney or urinary bladder in the rodents and some other substances. IARC monographs on the evaluation of carcinogenic risk of chemicals to humans. International Agency for Research of Cancer, Lyon, France.

Anonymous. (2006). Infectious Diseases in Finland 2005. Publications of the National Public Health Institute. B 17/2006. 60 p.

Anriany, Y.A., Weiner, R.W., Johnson, J.A., de Rezende, C. & Joseph, S.W. (2001). *Salmonella enterica* serovar Typhimurium DT104 displays a rugose phenotype. *Applied and Environmental Microbiology*. 9, 4048–4056.

Anriany, Y.A, Sahu, S.N., Wessels, K.R., McCann, L.M. & Joseph, S.W. (2006). Alteration of the rugose phenotype in *waa*G and *ddh*C mutants of *Salmonella enterica* serovar Typhimurium DT205 is associated with inverse production of curli and cellulose. *Applied and Environmental Microbiology*. 7, 2002–2012.

Antikainen, J., Anton, L., Sillanpää, J. & Korhonen, T.K. (2002). Domains in the S-layer protein CbsA of *Lactobacillus crispatus* involved in adherance to collagens, laminin and lipoteichoic acids and in self-assembly. *Molecular Microbiology*. 46, 381–394.

van Asselt, E.D. & Zwietering, M.H. (2006). A systematic approach to determine global thermal inactivation parameters for various food pathogens. *International Journal of Food Microbiology*. 107, 73–82.

Ayres, H.M., Furr, J.R. & Russell, A.D. (1993). A rapid method of evaluating permeabilizing activity against *Pseudomonas aeruginosa. Letters in Applied Microbiology.* 17, 149–151.

Ayres, H.M., Payne, D.N., Furr, J.R. & Russell, A.D. (1998). Use of the Malthus-AT system to assess the efficacy of permeabilizing agents on the activity of antimicrobial agents against *Pseudomonas aeruginosa*. *Letters Applied Microbiology*. 6, 422–426.

Bahulekar, R., Ayyangar, N.R. & Ponrathnam, S. (1991). Polyethyleneimine in immobilization of biocatalysts. *Enzyme and Microbiological Technology.* 13, 858–868.

Banin, E., Brady, K.M. & Greenberg, E.P. (2006). Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. *Applied and Environmental Microbiology*. 72, 2064–2069.

Bansal-Mutalik, R. & Gaikar, V.G. (2003). Cell permeabilization for extraction of penicillin acylase from *Escherichia coli* by reverse micellar solutions. *Enzyme and Microbiological Technology*. 32, 14–26.

Baylis, C.L. (2006). Enterobacteriacea. Chapter 22. In: Food Spoilage Microorganisms. Ed. Blackburn, C. de W. Woodhead Publishing. CRC Press. Pp. 579–623.

Bengoechea, J.A., Diaz, R. & Moriyón, I. (1996). Outer membrane differences between pathogenic and environmental *Yersinia enterocolitica* biogroups probed with hydrophobic permeants and polycationic peptides. *Infection and Immunity.* 64, 4891–4899.

Bengoechea, J.A., Frandenburg, K., Sydel, U., Diaz, R. & Moriyón, I. (1998). *Yersinia pseudotuberculosis* and *Yersinia pestis* show increased outer membrane permeability to hydrophobic agents which correlates with lipopolysaccharide acyl-chain fluidity. *Microbiology*. 144, 1517–1526.

Bengoechea, J.A., Brandenburg, K., Arraiza, M.D., Seydel, U., Skurnik, M. & Moriyón, I. (2003). Pathogenic *Yersinia enterocolitica* strains increase the outer membrane permeability in response to environmental stimuli by modulating lipopolysaccharide fluidity and lipid A structure. *Infection and Immunity*. 71, 2014–2021.

Beveridge, T.J. (2001). Use of the Gram stain in microbiology. *Biotechnic & Histochemistry*. 76, 111–118.

Bjornsdottir, K., Breidt, F. Jr. & McFeeters, R.F. (2006) Protective effects of organic acids on survival of *Escherichia coli* O157:H7 in acidic environments. *Applied and Environmental Microbiology*. 72, 660–664.

Bolshakova, A.V., Kiselyova, O.I. & Yaminsky, I.V. (2004). Microbial surfaces investigated using Atomic Force Microscopy. *Biotechnology Progress*. 20, 1615–1622.

Bos, M.P. & Tommassen, J. (2004). Biogenesis of the Gram-negative bacterial outer membrane. *Current Opinions in Microbiology*. 7, 610–616.

Bos, M.P. & Tommassen, J. (2005). Viability of capsule- and lipopolysaccharide-deficient mutant of *Neisseria meningitidis*. *Infection and Immunity*. 73, 6194–6197.

Boziaris, I.S. & Adams, M.R. (1999). Effect of chelators and nisin produced in situ on inhibition and inactivation of Gram negatives. *International Journal of Food Microbiology*. 53, 105–113.

Brennan, P.J. & Nikaido, H. (1995). The envelope of mycobacteria. *Annual Review of Biochemistry*. 64, 29–63.

Brul, S., Coote, P., Oomes, S., Mensonides, F., Hellingwerf, K. & Klis, F. (2002). Physiological actios of preservative agents: prospective of use of modern microbiological techniques in assessing microbial behaviour in food preservation. *International Journal of Food Microbiology*. 79, 55–64.

Cabeen, M.T. & Jacobs-Wagner, C. (2005). Bacterial cell shape. *Nature Reviews. Microbiology*. 3, 601–610.

Carroll, P., La Ragione, R.M., Sayers, A.R. & Woodward, M.J. (2004). The O-antigen of *Salmonella enterica* serotype Enteritidis PT4: a significant factor in gastrointestinal colonisation of young but not newly hatched chicks. *Veterinary Microbiology*. 102, 73–85.

Campanac, C., Pineau, L. Payard, A., Baziard-Mouysset, G. & Roques, C. (2002). Interactions between biocide cationic agents and bacterial biofilms. *Antimicrobial Agents Chemotherapy*. 46, 1469–1474.

Campos, M.A., Morey, P. & Bengoechea, J.A. (2006). Quinolones sensitize Gram-negative bacteria to antimicrobial peptides. *Antimicrobial Agents and Chemotherapy*. 50, 2361–2367.

Chen, C.Z. & Cooper, S.L. (2002). Interactions between dendrimer biocides and bacterial membranes. *Biomaterials*. 23, 3359–3368.

Chiu, C.-H., Su, L.-H. & Chu, C. (2004). *Salmonella enterica* serotype Chloraesuis: Epidemiology, pathogenesis, clinical disease and treatment. *Clinical Microbiology Reviews*. 2, 311–322.

Cogan, T.A., Jorgensen, F., Lappin-Scott, H.M., Benson, C.E., Woodward, M.J. & Humprey, T.J. (2004). Flagella and curli fimbriae are important for the growth of *Salmonella enterica* serovars in hen eggs. *Microbiology*. 150, 1063–1071.

Crispim, C.A. & Gaylarde, C.C. (2005). Cyanobacteria and Biodeterioration of Cultural Heritage: A Review. *Microbial Ecology*. 1, 1–9.

Daniel, R.A. & Errington, J. (2003). Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell. *Cell*. 113, 767–776.

Das, E., Gürakan, C. & Bayindirh, A. (2006). Effect of controlled atmosphere storage, modified atmosphere packaking and gaseous ozone treatment on the survival of *Salmonella* Enteritidis on cherry tomatoes. *Food Microbiology*. 23, 430–438.

Davey, M.E. & O'Toole, G.A. (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews*. 4, 847–867.

Davidson, P.M. (1997). Chemical preservatives and natural antimicrobial compounds. Chapter 29. In: Food Microbiology. Fundamentals and Frontiers. Eds. Doyle, M.P., Beuchat, L.R., & Montville, T.J. ASM Press. Washington, D.C. USA. Pp. 520–556.

Doktycz, M.J., Sullivan, C.J., Hoyt, P.R., Pelletier, D.A., Wu, S. & Allison, D.P. (2003). AFM imaging of bacteria in liquid media immobilized on gelatine coated mica surfaces. *Ultramicroscopy.* 97, 209–216.

Donlan, R.M. & Costerton, J.W. (2002). Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews.* 2, 167–193.

Doores, S. (1993). Organic acids. Chapter 4. In: Davidson, P.M. & Branen, A.L. Eds. Antimicrobials in Food. Marcel Dekker Ltd., New York, 2 ed. Pp. 95–136.

Dornieden, Th., Gorbushina, A.A. & Krumbein, W.E. (2000). Biodecay of cultural heritage as a space/time-related ecological situation – an evaluation of a series of studies. *International Biodeterioration Biodegradation*. 43, 261–270.

Drudy, D., O'Rourke, M., Murphy, M., Mullane, N.R., O'Mahony, R., Kelly, L., Fischer, M., Sanjag, S., Shannon, P., Wall, P., O'Mahony, M., Whyte, P. & Fanning, S. (2006). Characterization of a collection of *Enterobacter sakazakii* isolates from environmental and food sources. *International Journal of Food Microbiology*. 110, 127–134.

Duffy, E.A., Lucia, L.M., Kells, J.M., Castillo, A., Pillai, S.D. & Acuff, G.R. (2005). Concentrations of *Escherichia coli* and genetic diversity and antibiotic resistance profiling of *Salmonella* isolated from irrigation water, packing shed equipment, and fresh produce in Texas. *Journal of Food Protection*. 68, 70–79.

Dufrene, Y.F. (2001). Application of atomic force microscopy to microbial surfaces: from reconstituted cell surface layers to living cells. *Micron*. 32, 153–165.

Edelson-Mammel, S., Porteous, M.K. & Buchanan, R.L. (2006). Acid resistance of twelve strains of *Enterobacter sakazakii*, and the impact of habituating the cells to an acidic environment. *Journal of Food Science*. 71, M210–M207.

EFSA, European Food Safety Authority. (2006). Trends and source of zoonoses, zoonotic agents and antimicrobial resistance in the European Union in 2004. *EFSA Journal*. 310, 10: 23.95.

El-Kosasy, Am.M. (2006). Potentiometric assessment of Gram-negative bacterial permeabilization of tobramycin. *Journal of Pharmaceutical and Biomedical Analysis*. 42, 389–394.

Ellison III, R.T., Giehl, T.J. & LaForce, F.M. (1988). Damage of the outer membrane of enteric Gram-negative bacteria by lactoferrin and transferrin. *Infection and Immunity*. 56, 2774–2781.

Erbel, P.J.A., Barr, K., Gao, N., Gerwig, G.J., Rick, P.D. & Gardner, K.H. (2003). Identification and biosynthesis of cyclic enterobacterial common antigen in *Escherichia coli*. *Journal of Bacteriology*. 185, 1995–2004.

Erridge, C., Bennett-Guerrero, E. & Poxton, I.R. (2002). Structure and function of lipopolysaccharides. *Microbes and Infection*. 4, 837–851.

Fayol-Messoudi, D., Berger, C.N., Coconnier-Polter, M.H., Lievin-Le Moal, V. & Servin, A.L. (2005). pH-, lactic acid-, and non-lactic acid-dependent activities of probiotic lactobacilli against *Salmonella enterica* serovar Typhimurium. *Applied and Environmental Microbiology*. 71, 6008–6013.

Finch, J.E. & Brown, M.R. (1975). The influence of nutrient limitation in a chemostat on the sensitivity of *Pseudomonas aeruginosa* to polymyxin and EDTA. *Journal of antimicrobial and Chemotherapy*. 1, 379–386.

Fleet, G.H. (1999). Microorganisms in food ecosystems. *International Journal of Food Microbiology*. 50, 101–117.

Forsberg, L.S. & Carlson, R.W. (1998). The structures of the lipopolysaccharides from *Rhizobium etli* strains CE358 and CE359. *The Journal of Biological Chemistry*. 273, 2747–2757.

Foster, J.W. (2004). *Escherichia coli* acid resistance: Tales of an amateur acidophile. *Nature Reviews. Microbiology*. 2, 898–907.

Fredriksson-Ahomaa, M., Stolle, A. & Korkeala, H. (2006). Molecular epidemiology of *Yersinia enterocolitica* infections. *FEMS Immunology and Medical Microbiology*. 47, 315–329.

Fresno, S., Jiménez, N., Izquierdo, L., Merino, S., Corsaro, M.M., De Castro, C., Parrilli, M., Naldi, T., Regué, M. & Tomás, J.M. (2006). The ionic interaction of *Klebsiella pneumoniae* K2 capsule and core lipopolysaccharide. *Microbiology*. 152, 1807–1818.

Fresno, S., Jiménez, N., Canals, R., Merino, S., Corsaro, M.M., Lanzetta, R., Parrilli, M., Pieretti, G., Regué, M. & Tomás, J.M. (2007). A second galacturonic acid transferase is required for core lipopolysaccharide biosynthesis and complete capsule association with the cell surface in *Klebsiella pneumoniae*. *Journal of Bacteriology*. 189, 1128–1137.

Frirdich, E. & Whitfield, C. (2005). Lipopolysaccharide inner core oligosaccharide structure and outer membrane stability in human pathogens belonging to the *Enterobacteriaceae*. *Journal of Endotoxin Research*. 11, 133–144.

Gaboriaud, F., Bailet, S., Dague, E. & Jorand, F. (2005). Surface structure and nanomechanical properties of *Shewanella putrefaciens* bacteria at two pH values (4 and 10) determined by Atomic Force Microscopy. *Journal of Bacteriology*. 11, 3864–3868.

Ganesh Kumar, C. & Anand, S.K. (1998). Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology*. 42, 9–27.

Garcia-Martin, O. (2004). Influence of environmental factors and human activity on the presence of *Salmonella* serovars in a marine environment. *Applied and Environmental Microbiology*. 70, 2089–2097.

Gasem, M.H., Dolmans, W.M.V., Keuter, M. & Djokomoeljanto, R. (2002). Poor food hygiene and housing as risk factors for typhoid fever in Semerang, Indonesia. *Tropical Medicine and International Health*. 6, 484–490.

Gaynor, E.C., Wells, D.H., MacKichan, J.K. & Falkow, S. (2005). The *Campylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes. *Molecular Microbiology*. 56(1), 8–27.

Gee, J.M., Wortley, G.M., Price, K.R. & Johnson, L.T. (1999). Use of an in vitro model to assess factors influencing the effect of legume saponins on the intestinal epithelium. COST 98. Proceedings of the sixth scientific workshop in Rome. 8.–10.5.1997. Ed. Carnovale, E. European Commission. Pp. 139–144.

Gibson, D.L., White, A.P., Snyder, S.D., Martin, S., Heiss, C., Azadi, P., Surette, M. & Kay, W.W. (2006). *Salmonella* produces an O-antigen capsule regulated by AgfD and important for environmental persistence. *Journal of Bacteriology*. 188, 7722–7730.

Gilbert, P. & McBain, A.J. (2003). Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. *Clinical Microbiology Reviews*. 16, 189–208.

Glukhov, E., Stark, M., Burrows, L.L. & Deber, C.M. (2005). Basis for selectivity of cationic antimicrobial peptides for bacterial versus mammalian membranes. *Journal of Biological Chemistry*. 280, 33960–33967.

Gram, L., Ravn, L., Rasch, M., Bartholin Bruhn, J., Christensen, A.B. & Givskov, M. (2002). Food spoilage- interactions between food spoilage bacteria. *International Journal of Food Microbiology*. 78, 79–97.

Groisman, E.A. & Ochman, H. (1997). How *Salmonella* became a pathogen. *Trends in Microbiology*. 5, 343–349.

Gronow, S. & Brade, H. (2001). Lipopolysaccharide biosynthesis: which steps do bacteria need to survive? *Journal of Endotoxin Research*. 7, 3–23.

Guard-Bouldin, J., Gast, R.K., Humprey, T.J., Henzler, D.J., Morales, C. & Coles, K. (2004). Subpopulation characterictis of egg-contaminating *Salmonella enterica* serovar Enteritidis as defined by the lipopolysaccharide O chain. *Applied and Environmental Microbiology*. 70, 2756–2763.

Guard-Petter, J. (2001). The chicken, the egg and *Salmonella enteritidis*. *Environmental Microbiology*. 3, 421–430.

Guerrero, R., Piqueras, M. & Berlanga, M. (2002). Microbial mats and the search for minimal ecosystems. *International Microbiology.* 5, 177–188.

Gunn, J.S. (2001). Bacterial modification of LPS and resistance to antimicrobial peptides. *Journal of Endotoxin Research.* 7, 57–62.

Gunn, J.S., Lim, K.B., Krueger, J., Kim, K., Guo, L., Hackett, M. & Miller, S.I. (1998). PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Molecular Microbiology*. 27, 1171–1182.

Gunn, J.S., Ryan, S.S., van Velkinburgh, J.C., Ernst, R.K. & Miller, S.I. (2000). Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar Typhimurium. *Infection and Immunity*. 68, 6139–6146.

Guo, L. Lim, K.B., Gunn, J.S., Bainbridge, B., Darveau, R.P., Hackett, M. & Miller, S.I. (1997). Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes phoP-phoQ. *Science*. 276, 250–253.

Guo, L., Lim, K., B., Poduje, C.M., Daniel, M., Gunn, J.S., Hackett, M. & Miller, S.I. (1998). Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell*. 95, 189–198.

Gutsmann, T., Hagge, S.O., David, A., Roes, S., Böhling, A., Hammer, M.U. & Seydel, U. (2005). Lipid-mediated resistance of Gram-negative bacteria against various pore-forming antimicrobial peptides. *Journal of Endotoxin Research*. 11, 167–173.

Hancock, R.E.W. (1998). Resistance mechanisms in *Pseudomonas aeruginosa* and other non-fermentative gram-negative bacteria. *Clinical Infectious Diseases.* 27(Suppl.I), S93–99.

Hancock, R.E.W. & Wong, P.G.W. (1984). Compounds which increase the permeability of the *Pseudomonas aeruginosa* outer membrane. *Antimicrobial Agents and Chemoptherapy*. 26, 48–52.

Harrington, S.M., Dudley, E.G. & Nataro, J.P. (2006). Pathogenesis of enteroaggregative *Escherichia coli* infection. *FEMS Microbiology Letters*. 254, 12–18.

Helander, I.M., Lindner, B., Brade, H., Altmann, K., Lindberg, A.A., Rietschel, E.T. & Zahringer, U. (1988). Chemical structure of the lipopolysaccharide of *Haemophilus influenzae* strain I-69 Rd-/b+. Description of a novel deep-rough chemotype. *European Journal of Biochemistry*. 177, 483–492.

Helander, I.M., Kilpeläinen, I. & Vaara, M. (1994). Increased substitution of phosphate groups and lipid A of the polymyxin resistant pmrA mutants of *Salmonella typhimurium*: a 31P-NMR study. *Molecular Microbiology*. 11, 481–487.

Helander, I.M., Mäkelä, P.H., Westphal, O. & Rietschel, E.T. (1996). Lipopolysaccharides. In: Encyclopedia of Molecular Biology and Molecular Medicine, Vol. 3. Ed. R.A. Meyers. VCH, Weinheim, Germany. Pp. 462–471.

Helander, I.M., Alakomi, H.-L., Latva-Kala, K. & Koski, P. (1997a). Polyethyleneimine is an effective permeabilizer of gram-negative bacteria. *Microbiology*. 143, 3193–3199.

Helander, I.M., Kilpeläinen, I. & Vaara, M. (1997b). Phosphate groups in lipopolysaccharides of *Salmonella typhimurium rfaP* mutants. *FEBS Letters*. 409, 457–460.

Helander, I.M., von Wright, A. & Mattila-Sandholm, T. (1997c). Potential of lactic acid bacteria and novel antimicrobials against gram-negative bacteria. *Trends in Foods Science & Technology*. 8, 146–150.

Helander, I.M, Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Gorris, L.G.M. & von Wright, A. (1998a). Characterization of the action of selected essential oil components on gram-negative bacteria. *Journal of Agricultural and Food Chemistry*. 46, 3590–3595.

Helander, I.M., Latva-Kala, K. & Lounatmaa, K. (1998b). Permeabilizing action of polyethyleneimine on *Salmonella typhimurium* involves disruption of the outer membrane and interactions with lipopolysaccharide. *Microbiology*. 144, 385–390.

Helander, I.M. & Mattila-Sandholm, T. (2000) Fluorometric assessment of Gram-negative bacterial permeabilization. *Journal of Applied Microbiology*. 88, 213–219.

Helander, I.M., Nurmiaho-Lassila, E.-L., Ahvenainen, R., Rhoades, J. & Roller, S. (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. *International Journal of Food Microbiology*. 71, 235–244.

Heumann, D. & Roger T. (2002). Initial responses to endotoxins and Gram-negative bacteria. *Clinica Chimica Acta*. 323, 59–72.

Hirshfield, I.N., Terzulli, S. & O'Byrne, C. (2003). Weak organic acids: a panoply of effects on bacteria. *Science Progress*. 86, 245–269.

Hitchcock, P.J. & Brown, T.M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *Journal of Bacteriology*. 154, 269–277.

Holland, I.B. (2004). Translocation of bacterial proteins – an overview. *Biochemica et Biophysica Acta*. 1694, 5–16.

Holst, O. (1999). Chemical structure of the core region of lipopolysaccharides. In: Endotoxin in Health and Desiase. Eds. Brade, H., Opal, S.M., Vogel, S.N. & Morrison, D.C. New York/Basel. Marcel Dekker. Pp. 115–154.

Holt, S.C. & Beveridge, T.J. (1982). Electron microscopy: its development and application to microbiology. *Canadian Journal of Microbiology*. 28, 1–53.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. & Williams, S.T. (1994). Bergey's Manual of Determinative Bacteriology. Ninth edition. Williams & Wilkins. Baltimore, Maryland. 787 p.

Holzapfel, W.H., Geisen, R. & Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology*. 24, 343–362.

Huis in't Veld, J.H.J. (1996). Microbiological and biochemical spoilage of foods: an overwiev. *International Journal of Food Microbiology*. 33, 1–18.

Hukari, R., Helander, I.M & Vaara, M. (1986) Chain length heterogeneity of lipopolysaccharide released from *Salmonella typhimurium* by ethylenediaminetetraacetic acid or polycations. *European Journal Biochemistry*. 154, 673–676.

Humphrey, T. (2004). *Salmonella*, stress responses and food safety. *Nature Reviews. Microbiology*. 2, 504–509.

Isonhood, J.H. & Drake, M. (2002). *Aeromonas* species in foods. *Journal of Food Protection*. 65, 575–582.

Jackman, J.E., Fierke, C.A., Tumey, L.N., Pirrung, M., Uchiyama, T., Tahir, H., Hindsgaul, O. & Raetz, C.R.H. (2000). Antimicrobial agents that target lipid A biosynthesis in Gram-negative bacteria. *Journal of Biological Chemistry*. 15, 11002–11009.

Johnston, M.D., Hanlon, G.W., Denyer, S.P. & Lambert, R.J.W. (2003). Membrane damage to bacteria caused by single and combined biocides. *Journal of Applied Microbiology*. 94, 1015–1023.

Jorgensen, F., Leach, S., Wilde, S.J., Davies, A., Steward, G.S.A.B. & Humphrey, T. (2000). Invasiveness in chickens, stress resistance and RpoS satus of wild-type *Salmonella enterica* subsp. *enterica* serovar Typhimurium definitive type 104 and serovar Enteritidis phage type 4 strains. *Microbiology*. 146, 3227–3235.

Kanipest, M.I., Lin, S., Cotters, R.J. & Raetz, C.R.H (2001). Ca^{2+} -induced phosphoethanolaminen transfer to the outer 3-deoxy-D-manno-octulosonic acid moiety of *Escherichia coli* lipopolysaccharide. *Journal of Biological Chemistry.* 276, 1156–1163.

Kaper, J.B., Nataro, J.P. & Mobley, L.T. (2004). Pathogenic *Esherichia coli*. *Nature reviews. Microbiology*. 2, 123–140.

Kauffmann, S.H., Raupach, B. & Finlay, B.B. (2001). Introduction: microbiology and immunology: lessons learned from *Salmonella*. *Microbes and Infection*. 3, 1177–1181.

Kawahara, K., Seydel, U., Matsuura, M., Danbara, H., Rietschel, E. Th. & Zähringer, U. (1991). Chemical structure of glycosphingolipids isolated from *Sphingomonas paucimobilis*. *FEBS Letters*. 292, 107–110.

de Keersmaecker, S.C., Verhoeven, T.L., Desair, J., Marchal, K., Vanderleyden, J. & Nagy, I. (2006). Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. *FEMS Microbiological Letters*. 259, 89–96.

Knirel, Y.A, Bystrova, O.V., Kocharova, N.A., Zähringer, U. & Pier, G.B. (2006). Conserved and variable structural features in the lipopolysaccharide of *Pseudomonas aeruginosa*. *Journal of Endotoxin Research*. 12, 324–336.

Kolari, M., Nuutinen, J., Rainey, F.A. & Salkinoja-Salonen, M. (2003). Colored moderately thermophilic bacteria in paper-machine biofilms. *Journal of Industrial Microbiology and Biotechnology*. 30, 225–238.

Kooistra, O., Bedoux, G., Brecker, L., Lindner, B., Sánchez Carballo, P., Haras, D. & Zähringer, U. (2003). Structure of highly phosphorylated lipopolysaccharide core in the Δ*algC* mutants derived from *Pseudomonas aeruginosa* wild-type strains PAO1 (serogroup O5) and PAC1R (serogroup O3). *Carbohydrate Research*. 338, 2667–2677.

Kotra, L.P., Amro, N.A., Liu, G.Y. & Mobashery, S. (2000). Visualizing bacteria at high resolution. *ASM News*. 66, 675–681.

Lahtinen, T., Kosonen, M., Tiirola, M., Vuento, M. & Oker-Blom, C. (2006). Diversity of bacteria contaminating paper machines. *Journal of Industrial Microbiology and Biotechnology*. 33, 734–740.

Lazdunski, A.M., Ventre, I. & Sturgis, J.N. (2004). Regulatory circuits and communication in Gram-negative bacteria. *Nature Reviews*. 2, 581–591.

Leistner, L. (2000). Basic aspects of food preservation by hurdle concept. *International Journal of Food Microbiology*. 55, 181–186.

Leive, L. (1965). Release of lipopolysaccharide by EDTA treatment of *E. coli*. *Biochemical and Biophysical Research Communications.* 21, 290–296.

Leive, L. (1974) The barrier function of gram-negative encelope. *Annals of the New York Academy of Science.* 235, 109–127.

Levy, S.B. (2002). Active efflux, a common mechanisms for biocide and antibiotic resistance. *Journal of Applied Microbiology Symposium Supplement*. 92, 65S–71S.

Liebana, E., Garcia-Migura, L., Clouting, C., Clifton-Hadley, F.A., Breslin, M. & Davies, R.H. (2003). Molecular fingerprinting evidence of the contribution of wildlife vectors in the maintenance of *Salmonella* Enteritidis infection in layer farms. *Journal of Applied Microbiology*. 94, 1024–1029.

Little, C.L. & Knochel, S. (1994). Growth and survival of *Yersinia enterocolitica*, *Salmonella* and *Bacillus cereus* in brie stored at 4, 8 and 20 degrees Celsius. *International Journal of Food Microbiology*. 24, 137–145.

Loh, B., Grant, C. & Hancock, R.E.W. (1984). Use of the fluorescent probe 1-*N*-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer memebrane of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*. 26, 546–551.

Loughlin, M.F., Jones, M.V. & Lambert, P.A. (2002). *Pseudomonas aeruginosa* cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. *Journal of Antimicrobial and Chemotherapy.* 49, 631–639.

Maillard, J.Y. (2002). Bacterial target sites for biocide action. *Journal of Applied Microbiology. Symposium Supplement*. 90, 16S–27S.

Marcus, S.L., Brumell, J.H., Pfeifer, C.G. & Finlay, B.B. (2000). *Salmonella* pathogenicity islands: big virulence in small packages. *Microbes and Infection*. 2, 145–156.

Martinez-Urtaza, J., Saco, M., de Novoa, J., Perez-Pineiro, P., Peiteado, J., Lozano-Leon, A. & Garcia-Martin, O. (2004). Influence of environmental factors and human activity on the presence of *Salmonella* serovars in a marine environment. *Applied and Environmental Microbiology*. 70, 2089–2097.

Matsumoto, K., Kusaka, J., Nishibori, A. & Hara, H. (2006). Lipid domains in bacterial membranes. *Molecular Microbiology*. 61, 1110–1117.

McMahon, M.A.S., Xu, J., Moore, J.E., Blair, I.S. & McDowell, D.A. (2007). Environmental stress and antibiotic resitance in food-related pathogens. *Applied and Environmental Microbiology*. 73, 211–217.

Mdluli, K.E., Witte, P.R., Kline, T., Barb, A.W., Erwin, A.L., Mansfield, B.E., McClerren, A.L., Pirrung, M.C., Tumey, L.N., Warrener, P., Raetz, C.R. & Stover, C.K. (2006). Molecular validation of LpxC as and antibacterial drug target in *Pseudomonas aeruginosa. Antimicrobial agent and Chemotherapy*. 50, 2178–2184.

Meincken, M., Holroy, D.L. & Rautenbach, M. (2005). Atomic force microscopy study of the effect of antimicrobial peptides on the cell envelope of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy.* 49, 4085–4092.

Meroueh, S.O., Bencze, K.Z., Hesek, D., Lee, M., Fisher, J.F., Stemmler, T.L., & Mobashery, S. (2006). Three-dimensional structure of the bacterial cell wall peptidoglycan. *PNAS*. 12, 4404–4409.

Merrell, D.S. & Falkow, S. (2004). Frontal and stealth attack strategies in microbial pathogenesis. *Nature*. 430, 250–256.

le Minor, L. (1999). The genus *Salmonella*. Chapter in Prokaryotes. Release 3.0. Springer-Verlag, New-York. LCC.

Monack, D.M., Mueller, A. & Falkow, S. (2004). Persistent bacterial infections: the interface of the pathogen and the host immune system. *Natures Review in Microbiology*. 2, 747–765.

Munsch-Alatossava, P. & Alatossava, T. (2006). Phenotypic characterization of raw milk-associated psychrotrophic bacteria. *Microbiological Research*. 161, 334–346.

Nakimbugwe, D., Masschalck, B., Atanassova, M., Zewdie-Bosüner, A. & Michiels, C.W. (2006). Comparison of bactericidal activity of six lysozymes at atmospheric pressure and under high hydrostatic pressure. *International Journal of Food Microbiology*. 108, 355–363.

Nikaido, H. (1996a). Multidrug efflux pumps of gram-negative bacteria. *Journal of Bacteriology*. 178, 5853–5859.

Nikaido, H. (1996b). Outer membrane. Chapter 5. In: *Escherichia coli* and *Salmonella*. Cellular and Molecular Biology. Second ed. Vol. 1. Ed. in chief Neidhardt, F.C. ASM Press. Washington, D.C. U.S.A. Pp. 29–47.

Nikaido, H. (1999). Microdermatology: cell surface in the interaction of microbes with the external world. *Journal of Bacteriology*. 181, 4–8.

Nikaido, H. (2001). Preventing drug access to targets; cell surface permeability barriers and active efflux in bacteria. *Seminars in Cell and Development Biology*. 12, 215–223.

Nikaido, H. (2003). Molecular basics of bacterial outer membrane permeability revisited. *Microbiology Molecular Biology Reviews*. 4, 593–656.

Nikaido, H. & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews*. 49, 1–32.

Niskanen, T., Johansson, T., Kuusi, M., Raahenmaa, M., Siitonen, A. & Tuominen, P. (2006). Foodborne and waterborne outbreaks in Finland 2005. Evira Publications 2/2006. Helsinki: Finnish Food Safety Authority. (In Finnish.) 52 p.

Nohynek, L., Alakomi, H.-L., Kähkönen, M.P., Heinonen, M., Helander, I.M., Oksman-Caldentey, K.M. & Puupponen-Pimiä, R.H. (2006). Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. *Nutrition and Cancer.* 54, 18–32.

Nummila, K., Kilpeläinen, I., Zahringer, U., Vaara, M. & Helaner, I. (1995). Lipopolysaccharides of polymyxin B-resistant mutants of *Escherichia coli* are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. *Molecular Microbiology*. 16, 271–278.

Ohmizo, C., Yata, M. & Katsu, T. (2004). Bacterial cytoplasmic membrane permeability assay using ion-selective electrodes. *Journal of Microbiological Methods*. 59, 173–179.

Olsen, S.J., Bishop, R., Brenner, F.W., Roels, T.H., Bean, N., Tauxe, R.V. & Slutsker, L. (2002). The changing epidemiology of Salmonella: Trends in serotypes isolated from humans in the United States, 1987–1997. *The Journal of Infectious Diseases*. 183, 73–761.

Olsthoorn, M.M.A., Petersen, B.O., Schlect, S., Haverkamp, J., Bock, K., Thomas-Oates, J.E. & Holst, O. (1998). Identification of a novel core type in *Salmonella* lipopolysaccharide. *The Journal of Biological Chemistry.* 273, 3817–3829.

Orlov, D.S., Nguyen, T. & Lehrer, R.I. (2002). Potassium release, a useful tool for studying antimicrobial peptides. *Journal of Microbiological Methods*. 49, 325–328.

O'Toole, G., Kaplan, H.B. & Kolter, R. (2000). Review. Biofilm formation as microbiological development. *Annual Reviews in Microbiology*. 54, 49–79.

Pang, T., Levine, M.M., Ivanoff, B., Waln, J. & Finlay, B.B. (1998). Typhoid fever – important issues still remain. *Trends in Microbiology*. 4, 131–133.

Parker, C.T., Leibana, E., Henzler, D.J. & Guard-Petter, J. (2001). Lipopolysaccharide O-chain microheterogeneity of *Salmonella* serotypes Enteritidis and Typhimurium. *Environmental Microbiology*. 3, 332–342.

Paulus, W. (1993). Microbicides for the protection of materials – A handbook. Chapman & Hall. London. UK. 496 p.

Piddock, L.J.V. (2006). Multidrug-resistance efflux pumps – not just for resistance. *Nature Reviews. Microbiology.* 4, 629–636.

Plym Forshell, L. & Wierup, M. (2006). Salmonella contamination: a significant challenge to the global marketing of animal food products. *Scientific and Technical Review*, *OIE. World Organization for animal health.* 25, 541–554.

Preston, A., Mandrell, R.E., Gibson, B.W. & Apicella, M.A. (1996). The lipooligosaccharides of pathogenic gram-negative bacteria. *Critical Reviews Microbiology*. 22, 139–180.

Prigent-Combaret, C., Prensier, G., Le Thi, T.T., Vidal, O., Lejeune, P. & Dorel, C. (2000). Development of pathway for biofilm formation in curli-producing *Escherichia coli* strains: role of flagella, curli and colonic acid. *Environmental Microbiology*. 2, 450–464.

Poole, K. (2002). Outer membranes and efflux: The path to multidrug resistance in Gram-negative bacteria. *Current Pharmaceutical Biotechnology*. 3, 77–98.

Popoff, M.Y., Bockemühl, J. & Gheesling, L.L. (2004). Supplement 2002 (no. 46) to the Kauffman-White scheme. *Research in Microbiology*. 155, 568–570.

Puupponen-Pimiä, R., Nohynek, L., Alakomi, H.-L. & Oksman-Caldentrey, K. (2005). The action of berry phenolics against human intestinal pathogens. *Biofactors*. 23, 243–251.

Raaska, L., Alakomi, H.-L., Salkinoja-Salonen, M. & Mattila-Sandholm, T. (1999). Antagonistic activity of *Staphylococcus* siderophores and chemical biocides against *Bacillus subtilis* in a paper-machine environment. *Journal of Industrial Microbiology & Biotechnology.* 22, 27–32.

Raetz, C.R.H. (1996). Bacterial lipopolysaccharide. Chapter 69. In: *Escherichia coli* and *Salmonella*. Cellular and Molecular Biology. Second ed. Vol. 1. Ed. in chief Neidhardt, F.C. ASM Press. Washington, D.C. U.S.A. Pp. 1035–1063.

Raetz, C.R.H. & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annual Review Biochemistry*. 71, 635–700.

Raivio, T.L. (2005). Envelope stress responses and Gram-negative bacterial pathogenesis. *Molecular Microbiology*. 56, 1119–1128.

Rajamäki, T., Alakomi, H.-L., Ritvanen, T., Skyttä, E., Smolander, M. & Ahvenainen, R. (2006). Application of electronic nose for quality assessment of modified atmosphere packaged poultry meat. *Food Control*. 17, 5–13.

Raupach, B. & Kauffman, S.H.E. (2001). Immune responses to intracellular bacteria. *Current Opinion in Immunology*. 1, 417–428.

Rebell, R., Ernst, R.K., Gowen, B.B., Miller, S.I. & Hinnebush, B.J. (2004). Variation in lipid A structure in the pathogenic yersinia. *Molecular Microbiology*. 52, 1363–1373.

Reij, M.W. & Den Aantrekker, E.D. (2004). ILSI Europe Risk Analysis in Microbiology Task Force. Recontamination as a source of pathogens in processed foods. *International Journal of Food Microbiology*. 91, 1–11.

Reynolds, C.M., Ribeiro, A.A., McGrath, S.C., Cotter, R.J., Raetz, C.R.H. & Trent, M.S. (2006). An outer membrane enzyme encoded by *Salmonella typhimurium lpxR* that removes the 3'-acyloxyacyl moiety of lipid A. *The Journal of Biological Chemistry*. 31, 21974–21987.

de Rezende, C.E., Anriany, Y., Carr, L.E., Jospeh, S.W. & Weiner, R.M. (2005). Capsular polysaccharide surrounds smooth and rugose types of *Salmonella enterica* serovar Typhimurium DT104. *Applied and Environmental Microbiology*. 71, 7345–7351.

Rick, P.D. & Silver, R.P. (1996). Enterobacterial common antigen and capsular polysaccharides. Chapter 9. In: *Escherichia coli* and *Salmonella*. Cellular and Molecular Biology. Ed. in Chief Niedhardt, F.C. Second ed. Vol. 1. ASM Press. Washington, D.C., U.S.A. Pp. 104–122.

Ricke, S.C. (2003). Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poultry Science*. 82, 632–639.

Robertson, E.B. & Firestone, M.K. (1992). Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. *Applied and Environmental Microbiology.* 58, 1284–1291.

Rokka, M., Eerola, S., Smolander, M., Alakomi, H.-L. & Ahvenainen, R. (2004). Monitoring of the quality of modified atmosphere packaged broiler chicken cuts stored in different temperature conditions. B. Biogenic amines as quality-indicating metabolites. *Food Control*. 15, 601–607.

Ross, R.P., Morgan, S. & Hill, C. (2002). Preservation and fermentation: past, present and future. *International Journal of Food Microbiology*. 79, 3–16.

Roth, L.A. & Keenan, D. (1971). Acid injury in *Escherichia coli. Canadian Journal of Microbiology*. 17, 1005–1008.

Rowley, G., Spector, M., Kormanec, J. & Roberts, M. (2006). Pushing the envelope: extracytoplasmic stress responses in bacterial pathogenesis. *Nature Reviews. Microbiology*. 4, 383–394.

Ruiz, N., Kahne, D. & Silhavy, T.J. (2006). Advances in understanding bacterial outer-membrane biogenesis. *Nature Reviews*. 4, 57–65.

Russell, A.D. (2002). Antibiotic and biocide resistance in bacteria: Introduction. *Journal of Applied Microbiology*. 9 Suppl, 1S–3S.

Saarela, M., Alakomi, H.-L., Suihko, M.-L., Maunuksela, L., Raaska, L. & Mattila-Sandholm, T. (2004). Heterotrophic microorganisms in air and biofilm samples from Roman catacombs with a special emphasis on actinobacteria and fungi. *International Biodeterioration Biodegradation*. 54, 27–37.

Sauli, I., Danuser, J., Geeraerd, A.H., van Impe, J.F., Rüfenacht, J., Bissig-Choisat, B., Wenk, C. & Stärk, K.D.C. (2005). Estimating the probability and level of contamination with *Salmonella* of feed for finishing pigs produced in

Switzerland – the impact of the production pathway. *International Journal of Food Microbiology*. 100, 289–310.

Scalbert, A. & Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *Journal of Nutrition*. 130, 2073S–2085S.

Scheffers, D.-J. & Pinho, M.G. (2005). Bacterial cell wall synthesis: New insights from localization studies. *Microbiology and Molecular Biology Reviews*. 4, 585–607.

Schleifer, K.H., Leuteritz, M., Weiss, N., Ludwig, W., Kirchhof, G. & Seidel-Rufer, H. (1990). Taxonomic study of anaerobic, Gram-negative, rod-shaped bacteria from breweries: emended description of *Pectinatus cerevisiiphilus* and description of *Pectinatus frisingensis* sp. nov., *Selenomonas lacticifex* sp. nov., *Zymophilus raffinosivorans* gen. nov., sp. nov., and *Zymophilus paucivorans* sp. nov. *International Journal of Systematic Bacteriology.* 40, 19–27.

Schneitz, C., Nuotio, L., Mead, G. & Nurmi, E. (1992). Competitive exclusion in the young bird: challenge models, administration and reciprocal protection. *International Journal of Food Microbiology.* 15, 241–244.

Schneitz, C. & Renney, D.J. (2003). Effect of a commerical competitive exclusion product on the colonization of *Salmonella infantis* in day-old pheasant chicks. *Avian Diseases*. 47, 1448–1451.

Schooling, S.R. & Beveridge, T.J. (2006). Membrane vesicles: an overlooked component of the matrices of biofilms. *Journal of Bacteriology*. 188, 5945–5957.

Schäffer, C. & Messner, P. (2005). The structure of secondary cell wall polymers: how Gram-positive bacteria stick their cell walls together. *Microbiology*. 151, 643–651.

Schär-Zammaretti, P. & Ubbink, J. (2003). Imaging of lactic acid bacteria with AFM – elesticity and adhesion maps and their relationship to biological and structural data. *Ultramicroscopy*. 97, 199–208.

Scott, J.R. & Zahner, D. (2006). Pili with strong attachments: Gram-positive bacteria do it differently. *Molecular Microbiology*. 62, 320–330.

Scotter, M.J. & Castle, L. (2004). Chemical interactions between additives in foodstuffs: a review. *Food Additives and Contaminants.* 21, 93–124.

Servin, A.L. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiological Reviews*. 28, 405–440.

Severn, W.B., Kelly, R.F., Richards, J.C. & Whitfield, C. (1996). Structure of the core oligosaccharide in the serotype O8 lipopolysaccharide from *Klebsiella pneumoniae. Journal of Bacteriology.* 178, 1731–1741.

Shelef, L.A. & Seiter, J.A. (1993). Indirect Antimicrobials. In: Davidson, P. M. & Branen, A.L. (ed.). Antimicrobials in Food (Chapter 15), Marcel Dekker Ltd., New York. 2 ed. Pp. 539–569.

Silla Santos, M.H. (1996). Biogenic amines: their importance in foods. *International Journal of Food Microbiology*. 29, 213–231.

Silvestro, L., Weiser, J.N. & Axelsen, P.H. (2000). Antibacterial and antimembrane activities of cecropin A in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*. 44, 602–607.

Simoes, M., Carvalho, H., Pereira, M.O. & Vieira, M.J. (2003). Studies on the behaviour of *Pseudomonas fluorescens* biofilms after Ortho-phthalaldehyde treatment. *Biofouling*. 19, 151–157.

Skurnik, M., Venho, R., Bengoechea, J.A. & Moriyon, I. (1999). The lipopolysaccharide outer core of *Yersinia enterocolitica* serotype O:3 is required for virulence and plays a role in outer membrane integrity. *Molecular Microbiology*. 31, 1443–1462.

Skurnik, M. & Bengoechea, J.A. (2003). The biosynthesis and biological role of lipopolysaccharide O-antigens of pathogenic *Yersiniae*. *Carbohydrate Research*. 338, 2521–2529.

Smolander, M., Alakomi, H.-L., Ritvanen, T., Vainionpää, J. & Ahvenainen, R. (2004). Monitoring of the quality of modified atmosphere packaged broiler chicken cuts stored in different temperature conditions A. Time-temperature indicators as quality-indicating tools. *Food Control*. 15, 217–229.

Snyder, D.S., Gibson, D., Heiss, C., Kay, W. & Azadi, P. (2006). Structure of a capsular polysaccharide from *Salmonella enteritidis*. *Carbohydrate Research*. 341, 2388–2397.

Solano, C.B., Garicia, J., Valle, C., Berasain, J.M., Ghigo, J.M., Gamazo, C. & Lasa, I. (2002). Genetic analysis of *Salmonella enterica* biofilm formation: critical role of cellulose. *Molecular Microbiology*. 43, 793–808.

Sørum, H. & L'Abée-Lund, T.M. (2002). Antibiotic resistance in food-related bacteria – a result of interfering with the global web of bacterial genetics. *International Journal of Food Microbiology*. 78, 43–56.

Steeghs, L., den Hartog, R., den Boer, A., Zomer, B., Roholl, P. & van der Ley, P. (1998). Meningitis bacterium is viable without endotoxin. *Nature*. 392, 449–450.

Stenberg, F., Chovanec, P., Maslen, S.L., Robinson, C.V., Ilag, L.L., von Heijne, G. & Daley, D.O. (2005). Protein complexes of the *Escherichia coli* envelopes. *Journal of Biological Chemistry*. 41, 34409–34419.

Su, L.-H., Chiu, C.-H., Chu, C. & Ou, J.T. (2004). Antimicrobial resistance in nontyphoid *Salmonella* serotypes: A global challenge. *Clinical Infectious Diseases*. 39, 546–551.

Sutherland, I.W. (1999). Microbial polysaccharide products. *Biotechnology & Genetic Engineering Reviews*. 16, 217–229.

Szacfranek, J., Kumirska, J., Czerwicka, M., Kunikozska, D., Dziadziuszko, H. & Glośnicka, R. (2006). Structure and heterogeneity of the O-antigen of *Salmonella* Agona lipopolysaccharide. *FEMS Immunology and Medical Microbiology*. 48, 223–236.

Tauxe, R.V. (2002). Emerging foodborne pathogens. *International Journal of Food Microbiology*. 78, 31–41.

Terebiznik, M.R., Vazquez, C.L., Torbicki, K., Banks, D., Wang, T., Hong, W., Blanke, S.R., Colombo, M.I. & Jones, N.L. (2006). *Helicobacter pylori* VacA toxin promotes bacterial intracellular survival in gastric epithelial cells. *Infection and Immunity*. 74, 6599–6614.

Tindall, B.J., Grimont, PA.D., Garrity, G.M. & Euzéby, J.P. (2005). Nomenclature and taxonomy of the genus *Salmonella. International Journal of Systematic and Evolutionary Microbiology*. 55, 521–524.

Trent, M.S., Stead, C.M., Tran, A.X. & Hankins, J.V. (2006) Diversity of endotoxin and its impact on pathogenesis. *Journal of Endotoxin Research.* 12, 205–223.

Träuble, H. & Overath, P. (1973) The structure of *Escherichia coli* membranes studied by fluorescence measurements of lipid phase transition. *Biochimica Biophysica Acta* 307, 491–512.

Ubbink, J. & Schär-Zammaretti, P. (2005). Probing bacterial interactions: integrated approaches combining atomic force microscopy and biophysical techniques. *Micron*. 36, 293–320.

Vaara, M. (1981). Increased outer membrane resistance to ethylenediaminetetraacetate and cations in novel lipid A mutants. *Journal of Bacteriology.* 148, 426–434.

Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiological Reviews*. 56, 395–411.

Vaara, M. (1999). Lipopolysaccharide and the permeability of the bacterial outer membrane. In: H. Brade, S.M. Opal, S.N. Vogel, D.C. Morrison (ed.), Endotoxin in Health and Disease, Marcel Dekker, Inc., New York and Basel. Pp. 31–38.

Vaara, M. & Jaakola, J. (1989). Sodium hexametaphosphate sensitizes *Pseudomonas aeruginosa*, several other species of *Pseudomonas* and

Escherichia coli to hydrophobic drugs. *Antimicrobial Agent and Chemotherapy.* 33, 1741–1747.

Vaara, M. & Porro, M. (1996). Group of peptides that act synergistically with hydrophobic antibiotics against Gram-negative bacteria. *Antimicrobial Agents and Chemotherapy*. 40, 1801–1805.

Vaara, M. & Vaara, T. (1983a). Polycationic sensitize enteric bacteria to antibiotics. *Antimicrobial Agents and Chemotherapy*. 24, 107–113.

Vaara, M. & Vaara, T. (1983b). Polycations as outer membrane destabilizing agents. *Antimicrobial Agents and Chemotherapy*. 24, 114–122.

Veldhuizen, E.J., Tjeerdsma-van Bokhoven, J.L., Zweijtzer, C., Burt, S.A. & Haagsman, H.P. (2006). Structural requirements for the antimicrobial activity of carvacrol. *Journal of Agricultural and Food Chemistry.* 54, 1874–1879.

Venter, P., Abraham, M., Lues, J.F.R. & Ivanov, I. (2006). The influenze of sanitizers on the lipopolysaccharide composition of *Escherichia coli*. *International Journal of Food Microbiology*. 111, 221–227.

Vineeta, N., Singh, V. & Makkar, S. (2001). Antimicrobial activity of dimercaptosuccinic acid (DMSA): a new chelating agent. *Journal of the Indian Society Pedodontics and Preventive Dentistry*. 4, 160–163.

Voetsch, A.C., van Gilder, T.J., Angulo, F.J., Farley, M.M., Shallow, S., Marcus, R., Cieslak, P.R., Deneen, V.C. & Tauxe, R.V. (2004). FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. *Clinical Infectious Diseases*. 38 Suppl 3, S127–S134.

Vollaard, A.M., Ali, S., van Asten, H.A., Widjaja, S., Visser, L.G., Surjadi, C. & van Dissel, J.T. (2004). Risk factors for typhoid and paratyphoid fever in Jakarta, Indonesia. *JAMA*. 291, 2607–2615.

Vollmer, W. & Höltje, J.-V. (2001). Morphogenesis of *Escherichia coli*. *Current Opinion in Microbiology*. 4, 625–633.

Wagenaar, J.A., Mevius, D.J. & Havelaar, A.H. (2006). *Campylobacter* in primary animal production and control strategies to reduce the burden of human campylobacteriosis. *Scientific and Technical Review*, *OIE. World Organization for animal health.* 25, 581–594.

Walker, J.J., Spear, J.R. & Norman, N.R. (2005). Geobiology of a microbial endolithic community in the Yellowstone geothermal environment. *Nature*. 434(7036), 1011–1014.

Walsh, A.G., Matewish, M.J., Burrows, L.L., Monteiro, M.A., Perry, M.B. & Lam, J.S. (2000). Lipopolysaccharide core phosphates are required for viability and intrinsic drug resistance in *Pseudomonas aeruginosa*. *Molecular Microbiology*. 35, 712–727.

Walsh, S.E., Maillard, J.-Y., Russell, A.D., Catrenich, C.E., Charbonneau, D.L. & Bartolo, R.G. (2003a). Development of bacterial resistance to several biocides and effects on antibiotic susceptibility. *Journal of Hospital Infection*. 55, 98–107.

Walsh, S.E., Maillard, J.-Y., Russell, A.D., Catrenich, C.E., Charbonneau, D.L. & Bartolo, R.G. (2003b). Activity and mechanisms of action of selected biocidal agents on Gram-positive and Gram-negative bacteria. *Journal of Applied Microbiology*. 94, 240–247.

Wang, E.T., Tan, Z.Y., Willems, A., Fernández-López, M., Reinhold-Hurek, B. & Martinez-Romero, E. (2002). *Sinorhizobium morelense* sp. nov., a *Leucaena leucocephala*-associated bacterium that is highly resistant to multiple antibiotics. *International Journal of Systematic and Evolutionary Microbiology*. 52, 687–1693.

Warscheid, Th. & Braams, J. (2000). Biodeterioration of stone: a review. *International Biodeterioration Biodegradation.* 46, 343–368.

White, A.P., Collins, S.K., Banser, P.A., Gibson, D.L., Paetzel, M., Strynadka, N.C.J. & Kay, W.W. (2001). Structure and characterization of AgfB from *Salmonella enteritidis* thin aggregative fimbria. *Journal of Molecular Biology*. 311, 735–749.

White, A.P., Gibson, D.L., Collinson, S.K., Banser, P.A. & Kay, W.W. (2003). Extracellular polysaccharide associated with thin aggregative fimbria of *Salmonella enterica* serovar Enteritidis. *Journal of Bacteriology.* 18, 5398–5407.

White, P.P., Gibson, D.L., Kim, W., Kay, W.W. & Surette, M.G. (2006). Thin aggregative fimbria and cellulose enhance long term survival and persistence of *Salmonella*. *Journal of Bacteriology*. 9, 3219–3227.

Wiese, A., Gutsmann, T. & Seydel, U. (2003). Towards antibacterial strategies: studies on the mechanisms of interactions between antimicrobial peptides and model membranes. *Journal of Endotoxin Research*. 9, 67–84.

Wirtanen, G., Salo, S., Helander, I.M. & Mattila-Sandholm, T. (2001). Microbiological methods for testing disinfectant efficiency on *Pseudomonas* biofilm. *Colloids and Surfaces. B Biointerfaces.* 20, 37–50.

Wu, M. & Hancock, R.E.W. (1999). Interaction of the cyclic antimicrobial cationic peptide bactenecin with the outer and cytoplasmic membranes. *The Journal of Biological Chemistry*. 274, 29–35.

Wu, T., McCandlish, A.C., Gronenberg, L.S., Chng, S.-S. Silhavy, T.J. & Kahne, D. (2006). Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. *PNAS*. 31, 11754–11759.

Yasudu, K., Ohmizo, C. & Katsu, T. (2003). Potassium and tetraphenylphosphonium ion-selective electrodes for monitoring changes in the permeability of bacterial outer and cytoplasmic membranes. *Journal of Microbiological Methods*. 54, 111–115.

Yethon, J.A., Heinrichs, D.E., Monteiro, M.A., Perry, M.B. & Whitfield, C. (1998). Involment of *waa*Y, *waa*Q, and *waa*P in the modification of *Escherichia coli* lipopolysaccharide and their role in the formation of a stable outer membrane. *The Journal of Biological Chemistry*. 273, 26310–26316.

Yethon, J.A. & Whitfield, C. (2001a). Lipopolysaccharide as a target for the development of novel therapeutics in gram-negative bacteria. *Current Drug Targets – Infectious Disorders*. 1, 91–106.

Yethon, J.A. & Whitfield, C. (2001b). Purification and characterization of WaaP from *Escherichia coli*, a lipopolysaccharide kinase essential for outer membrane stability. *Journal of Biological Chemisty*. 276, 5498–5504.

Young, K.D. (2006). The selective value of bacterial shape. *Microbiology and Molecular Biology Reviews.* 70, 660–703.

Zhao, T., Zhao, P., West, J.W., Bernard, J.K., Cross, H.G. & Doyle, M.P. (2006). Inactivation of enterohemorrhagic *Escherichia coli* in rumen content- or feces-contaminated drinking water for cattle. *Applied and Environmental Microbiology.* 72, 3268–3273.

Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W. & Römling, U. (2001). The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Molecular Microbiology*. 39, 1452–1463.

Zuber, B., Haenni, M., Ribeiro, T., Minning, K., Lopes, F., Moreillon, P. & Dubochet, J. (2006). Granular layer in the periplasmic space of Gram-positive bacteria and fine structures of *Enterococcus gallinarum* and *Streptococcus gordonii* septa revealed by cryo-electron microscopy of vitreous sections. *Journal of Bacteriology*. 188, 6652–6660.

Zähringer, U., Lindner, B. & Rietschel, E. Th. (1994). Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry*. 50, 211–276.

Åvall-Jääskeläinen, S. & Palva, A. (2005). *Lactobacillus* surface layers and their applications. *FEMS Microbiology Reviews*. 29, 511–529.

Appendix III of this publication are not included in the PDF version.
Please order the printed version to get the complete publication
(http://www.vtt.fi/publications/index.jsp)

PAPER I

Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane

In: Applied and Environmental Microbiology 2000.
Vol. 66, No. 5, pp. 2001–2005
Reprinted with permission from the publisher.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2000, p. 2001–2005 Vol. 66, No. 5
0099-2240/00/$04.00+0
Copyright © 2000, American Society for Microbiology. All Rights Reserved.

Lactic Acid Permeabilizes Gram-Negative Bacteria by Disrupting the Outer Membrane

H.-L. ALAKOMI, E. SKYTTÄ, M. SAARELA, T. MATTILA-SANDHOLM,
K. LATVA-KALA, AND I. M. HELANDER*

VTT Biotechnology, FIN-02044 VTT, Espoo, Finland

Received 5 October 1999/Accepted 17 January 2000

The effect of lactic acid on the outer membrane permeability of *Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium was studied utilizing a fluorescent-probe uptake assay and sensitization to bacteriolysis. For control purposes, similar assays were performed with EDTA (a permeabilizer acting by chelation) and with hydrochloric acid, the latter at pH values corresponding to those yielded by lactic acid, and also in the presence of KCN. Already 5 mM (pH 4.0) lactic acid caused prominent permeabilization in each species, the effect in the fluorescence assay being stronger than that of EDTA or HCl. Similar results were obtained in the presence of KCN, except for *P. aeruginosa*, for which an increase in the effect of HCl was observed in the presence of KCN. The permeabilization by lactic and hydrochloric acid was partly abolished by $MgCl_2$. Lactic acid sensitized *E. coli* and serovar Typhimurium to the lytic action of sodium dodecyl sulfate (SDS) more efficiently than did HCl, whereas both acids sensitized *P. aeruginosa* to SDS and to Triton X-100. *P. aeruginosa* was effectively sensitized to lysozyme by lactic acid and by HCl. Considerable proportions of lipopolysaccharide were liberated from serovar Typhimurium by these acids; analysis of liberated material by electrophoresis and by fatty acid analysis showed that lactic acid was more active than EDTA or HCl in liberating lipopolysaccharide from the outer membrane. Thus, lactic acid, in addition to its antimicrobial property due to the lowering of the pH, also functions as a permeabilizer of the gram-negative bacterial outer membrane and may act as a potentiator of the effects of other antimicrobial substances.

Lactic acid, as produced by lactic acid starter culture bacteria or as an additive to foods, functions as a natural antimicrobial having a generally recognized as safe status. As reviewed by Doores (8), lactic acid is able to inhibit the growth of many types of food spoilage bacteria, including gram-negative species of the families *Enterobacteriaceae* and *Pseudomonadaceae*. Among other organic acids, lactic acid is recognized as a biopreservative in naturally fermented products (25), and numerous applications for decontamination of meat by lactic acid have been described (7, 10, 22, 29, 32, 33). The antibacterial action of lactic acid is largely, but not totally, assigned to its ability in the undissociated form to penetrate the cytoplasmic membrane, resulting in reduced intracellular pH and disruption of the transmembrane proton motive force (25).

The relative efficacy of lactic acid against gram-negative bacteria is not unexpected considering that as a small water-soluble molecule lactic acid gains access to the periplasm through the water-filled porin proteins of the outer membrane (OM), as reviewed by Nikaido (18). The OM, however, functions as an efficient permeability barrier that is able to exclude macromolecules (such as bacteriocins or enzymes) and hydrophobic substances (i.e., hydrophobic antibiotics). The permeability barrier property of the OM is largely due to the presence of a specific lipopolysaccharide (LPS) layer on the membrane surface. LPS molecules consist of a lipid part, termed lipid A, and a hydrophilic heteropolysaccharide chain protruding outward and providing the cell with a hydrophilic surface (11). Certain external agents that either release LPS and other components from the OM or intercalate in the membrane can abolish the integrity of the OM. In both cases there is a concomitant loss of the permeability barrier function. Such agents are called permeabilizers (31); examples include EDTA, which chelates divalent cations that stabilize molecular interactions in the OM so that LPS is released, and polycations such as polyethyleneimine (12) or polymyxin B nonapeptide, which cause OM damage without LPS release. Permeabilizers as such need not be bactericidal or bacteriostatic to gram-negative cells but, by enabling other compounds to penetrate, an increased susceptibility to hydrophobic antibiotics, detergents, lysozyme, or bacteriocins is achieved. Accordingly, food-grade permeabilizers in combination with other antimicrobials would be ideal as part of the hurdle concept in inhibiting gram-negative spoilage bacteria and pathogens in food materials (13).

Roth and Keenan reported in 1971 (26) that lactic acid is able to cause sublethal injury to *Escherichia coli*, and similar properties have also been assigned to acetic acid (23); indirect evidence inferred that such injury involved disruption of the LPS layer. A permeabilizer function of lactic acid would not only be utilizable in decontamination procedures and in protective cultures but it would also provide a mechanistic explanation supporting the antimicrobial and health-promoting effects of probiotic lactic acid bacteria (28). We have investigated here the effects of lactic acid on the permeability properties of OM of three gram-negative bacterial species associated with food safety and food spoilage.

(Some of these results were presented at the 99th General Meeting of The American Society for Microbiology, Chicago, Ill., May 30 to June 3, 1999.)

MATERIALS AND METHODS

Chemicals. Lactic acid (mixture of D and L forms; pro analysis grade), potassium lactate, and Triton X-100 were from BDH (Poole, England); chicken egg white lysozyme (EC 3.2.1.17), HEPES, *n*-heptadecanoic acid methyl ester, 1-*N*-phenylnaphthylamine (NPN), and sodium dodecyl sulfate (SDS) were from Sigma-Aldrich (Steinheim, Germany); and EDTA was from Riedel-de-Haen

* Corresponding author. Mailing address: VTT Biotechnology, Tietotie 2, FIN-02044 VTT, Espoo, Finland. Phone: 358-9-456 4452. Fax: 358-9-455 2103. E-mail: ilkka.helander@vtt.fi.

TABLE 1. NPN uptake induced by acids

Strain (*n*)[a]	Relative fluorescence ± SD[b] at:							
	pH 3.6 ± 0.1		pH 4.0 ± 0.1				pH 7.2 ± 0.1	
	Lactic acid (10 mM)	HCl	Lactic acid (5 mM)	Lactic acid + $MgCl_2$	HCl	HCl + $MgCl_2$	EDTA (1 mM)	EDTA + $MgCl_2$
E. coli (7)[b]	357 ± 75	191 ± 22	288 ± 40	201 ± 31	138 ± 24	38 ± 6	40 ± 13	2 ± 1
E. coli + KCN (2)	ND	ND	379 ± 2	218 ± 35	145 ± 41	55 ± 10	62 ± 8	4 ± 1
P. aeruginosa (3)	373 ± 38	262 ± 16	377 ± 29	246 ± 24	280 ± 29	378 ± 1	273 ± 21	10 ± 5
P. aeruginosa + KCN (2)	ND	ND	397 ± 16	270 ± 8	346 ± 41	374 ± 33	343 ± 49	2 ± 2
Serovar Typhimurium (4)	529 ± 38	328 ± 100	531 ± 56	463 ± 41	288 ± 126	122 ± 64	99 ± 39	12 ± 3
Serovar Typhimurium + KCN (2)	496 ± 14	229 ± 104	480 ± 22	426 ± 35	147 ± 112	66 ± 36	67 ± 15	15 ± 2

[a] *n*, Number of independent experiments.
[b] The NPN uptake values for the cell controls subtracted from the total fluorescence values to obtain the values shown in the table were 99 ± 21, 70 ± 22 ($MgCl_2$), 116 ± 13 (KCN), and 87 ± 5 (KCN + $MgCl_2$) for *E. coli*; 124 ± 12, 107 ± 9 ($MgCl_2$), 171 ± 13 (KCN), and 129 ± 26 (KCN + $MgCl_2$) for *P. aeruginosa*; and 145 ± 22, 98 ± 16 ($MgCl_2$), 141 ± 18 (KCN), and 78 ± 11 (KCN + $MgCl_2$) for serovar Typhimurium.

(Seelze, Germany). Proteinase K (EC 3.4.21.64) and KCN were from Merck (Darmstadt, Germany). A stock solution of NPN (0.5 M) was prepared in acetone and diluted to 40 μM into 5 mM HEPES (pH 7.2) for the fluorometric assays. All materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Novex (San Diego, Calif.), including precast Tris-glycine gels (18% acrylamide).

Bacteria. *E. coli* ATCC 35150 (O157:H7), *Pseudomonas aeruginosa* ATCC 9027, and *Salmonella enterica* serovar Typhimurium SL696 (34) were cultivated in Luria-Bertani broth as described previously (12).

Permeability assay based on the uptake of NPN. NPN is a very hydrophobic probe whose quantum yield is greatly enhanced in a glycerophospholipid environment compared to an aqueous environment (30). Uptake of NPN by bacterial membrane(s) is manifested as fluorescence, and it indicates damage in the gram-negative bacterial OM, which normally is able to exclude hydrophobic substances (18). This permeability assay was recently adapted for the automated spectrofluorometer Fluoroskan (Labsystems, Helsinki, Finland), whereby fluorescent readings are made from microtiter plates (15). For these experiments, bacteria were grown to the mid-logarithmic phase of growth (optical density at 630 nm of 0.5 ± 0.02), deposited by centrifugation, and suspended into a half-volume of 5 mM HEPES buffer (pH 7.2). Such suspensions were then supplemented with lactic acid at 0.05 or 0.1% (wt/vol) corresponding to 5 mM and 10 mM, respectively, and causing the pH to drop to 4.0 ± 0.1 and 3.6 ± 0.1. Parallel suspensions were made in which pH was adjusted to the above values with hydrochloric acid. In assays involving KCN to de-energize cells, this salt was included in the buffers. The pH-adjusted suspensions (100 μl) were then pipetted into microtiter plate wells (Cliniplate Black, catalog no. 9502 867; Labsystems), which already contained 50 μl of pH-adjusted cell-free buffer and 50 μl of a 40 μM solution of NPN in buffer, yielding an end concentration of 10 μM NPN. In assays utilizing potassium lactate (10 mM), EDTA (1 mM), or $MgCl_2$ (5 mM), these were included in the cell-free buffer. Immediately after the cells were mixed with the other constituents, the plates were read for fluorescence in the Fluoroskan, using an excitation filter of 355 nm (half bandwidth, 38 ± 3 nm) and an emission filter of 405 nm (half bandwidth, 50 ± 5 nm). The fluorescence values were subtracted with the simultaneously recorded value of cell suspension in HEPES at pH 7.2 in the presence of 10 μM NPN. Four parallel wells of each sample were recorded, and three to seven independent assays were performed; experiments involving KCN were performed twice.

Bacteriolysis. Sensitization of bacteria to the action of lytic agents by acids or KCN was measured as described recently (12), utilizing turbidometric monitoring of cell lysis with the Multiskan MCC/340 spectrophotometer (Labsystems).

Release of LPS and phospholipid. The release of LPS from serovar Typhimurium was assayed by SDS-PAGE and by fatty acid analysis (gas chromatography of fatty acid methyl esters) of cell-free supernatants after treatment of the bacterial suspensions with either lactic acid, HCl, or EDTA. The protocols for these experiments were recently described in detail (14). The concentration of lactic acid in the release assay was 5 mM, yielding a pH value of 3.5 in the 10 mM Tris buffer initially adjusted to pH 7.2 by HCl. In parallel, cell suspensions in the above buffer were adjusted to pH 3.5 by HCl alone. Treatment with EDTA was at 1 mM at pH 7.2. From 10-ml suspensions, 8.6 ml of cell-free supernatant was taken for fatty acid analysis, and 0.5 ml was taken for SDS-PAGE. Both aliquots were freeze-dried before processing.

Statistical methods. For the NPN uptake values and the bacteriolysis values, the two-tailed unpaired Student's *t* test was used to determine differences; a P value of <0.05 was considered significant.

RESULTS

NPN uptake induced by acids. Table 1 summarizes the results of NPN uptake experiments with lactic acid, HCl, and EDTA, including the effect of addition of the $MgCl_2$ or the presence of KCN in the assay buffer. For all bacteria, lactic acid brought about a significantly higher NPN uptake than hydrochloric acid. The effect was seen already at 5 mM lactic acid (pH 4.0); only with *E. coli* was the NPN uptake further enhanced by the higher concentration of lactic acid (10 mM, pH 3.6). The strongest response to lactic acid was observed in serovar Typhimurium, but each test organism reacted more strongly to lactic acid than to the classical permeabilizer EDTA. The addition of an equimolar concentration of $MgCl_2$ together with lactic acid decreased the NPN uptake slightly but significantly for *E. coli* and *P. aeruginosa*; in serovar Typhimurium such an effect was insignificant. $MgCl_2$ also diminished the effect of HCl, but only in *E. coli*; surprisingly, *P. aeruginosa* reacted with higher uptake values to HCl in the presence of $MgCl_2$ than in its absence. The responses to EDTA differed characteristically among the three bacterial species, *P. aeruginosa* reacting most prominently and *E. coli* with the lowest figures; $MgCl_2$ abolished the effect in all cases. The above effects were generally similar in the presence of KCN; except for *P. aeruginosa*, for which the effect of HCl with KCN was enhanced to a level similar to that obtained by lactic acid. Potassium lactate at concentrations of up to 10 mM (pH 6.8 in the cell suspension) had no NPN uptake-enhancing activity on serovar Typhimurium (data not shown).

Effect of acids on bacteriolysis. To further investigate the permeabilizer effect of lactic acid, its effect on the sensitivity of bacteria toward lysozyme and the detergents SDS and Triton X-100 was measured. In parallel, similar assays with HCl (pH 3.6) and KCN (1 mM) were performed. The results are summarized in Table 2. Lactic acid had a strong sensitizing effect to SDS in each species; similar effects to the nonionic detergent Triton X-100 were also noted, especially in *P. aeruginosa*. This strain was also strongly sensitized by lactic acid to the lytic action of lysozyme. Hydrochloric acid brought about significantly weaker sensitizing effects than lactic acid to SDS in the enteric bacteria. However, *P. aeruginosa* was strongly affected; *P. aeruginosa* was also sensitized to Triton X-100 by HCl, but less so to lysozyme than by lactic acid. Although KCN had a slight effect on the SDS sensitivity of each species and also a minimal effect with Triton X-100 on *P. aeruginosa*, it was evident that de-energization of the bacterial cells did not have any major impact on their permeability properties.

Acids induce LPS release. Cell-free supernatants after treatment of serovar Typhimurium with lactic acid, HCl, or EDTA were processed for SDS-PAGE to investigate the possible release of LPS. A silver-stained gel showing the result is pictured in Fig. 1. Whereas very little LPS was present in the superna-

TABLE 2. Sensitization of gram-negative bacteria to lytic agents

Strain and lytic substance	Concn	Relative turbidity (%) at 4 min[a]			
		Control	Lactic acid (10 mM, pH 3.6)	HCl (pH 3.6)	KCN (1 mM, pH 7.2)
E. coli O157:H7					
Lysozyme	10 μg/ml	101 ± 2	99 ± 1	100 ± 1	100 ± 1
Triton X-100	0.1%	104 ± 1	102 ± 1	104 ± 1	103 ± 2
Triton X-100	1%	95 ± 1	92 ± 1	95 ± 1	95 ± 1
SDS	0.1%	102 ± 1	52 ± 11	102 ± 1	97 ± 2
SDS	1%	97 ± 1	47 ± 7	95 ± 1	83 ± 3
P. aeruginosa					
Lysozyme	10 μg/ml	99 ± 1	23 ± 1	53 ± 26	101 ± 1
Triton X-100	0.1%	102 ± 3	80 ± 1	55 ± 20	104 ± 1
Triton X-100	1%	98 ± 1	69 ± 1	45 ± 19	92 ± 1
SDS	0.1%	104 ± 2	18 ± 1	17 ± 1	101 ± 1
SDS	1%	101 ± 1	ND	ND	94 ± 1
Salmonella serovar Typhimurium					
Lysozyme	10 μg/ml	101 ± 1	102 ± 2	100 ± 1	102 ± 1
Triton X-100	0.1%	104 ± 3	101 ± 4	103 ± 1	104 ± 1
Triton X-100	1%	97 ± 1	91 ± 2	92 ± 2	94 ± 5
SDS	0.1%	102 ± 3	60 ± 10	94 ± 2	98 ± 1
SDS	1%	97 ± 2	53 ± 10	85 ± 3	77 ± 11

[a] ND, not determined.

tant of untreated cells, the supernatants of acid-treated suspensions yielded prominent ladder patterns characteristic of serovar Typhimurium smooth-type LPS. Based on visual estimation of the intensity of staining, the supernatant of lactic acid-treated bacteria contained more LPS than those derived from treatments by HCl or EDTA. The supernatants were also subjected to fatty acid analysis to obtain both qualitative and quantitative data on the released lipid material. Analysis results (Table 3) confirmed that lactic acid had been the most active acid with respect to LPS release, as indicated by the greatest sum of LPS-specific (35) fatty acids $C_{12:0}$, $C_{14:0}$, and $C_{3\text{-OH-}14:0}$. In addition to LPS, also other lipid material (glycerophospholipids) was released, represented by the unsaturated fatty acids found in the supernatants. However, LPS-specific fatty acids accounted for a greater proportion in the acid supernatants compared to that of the control, suggesting a preferential release of LPS.

DISCUSSION

The results presented here permit the conclusion that lactic acid is a potent OM-disintegrating agent, as evidenced by its ability to cause LPS release and to sensitize bacteria to detergents or lysozyme. Increase of the uptake of the hydrophobic probe NPN further suggests a permeabilizing action for lactic acid. Whereas acidity as adjusted by hydrochloric acid also brought about effects indicative of OM disruption, the direct effect of lactic acid as measured by the NPN uptake method was always stronger than that observed in HCl-treated bacteria at the same pH. Our data are thus in accord with and offer a potential mechanism for earlier findings that organic acids, including lactic acid, cause sublethal injury for gram-negative bacteria, as indicated by their decreased viability on bile salt-containing agar (23, 25, 26).

Disruption of the OM by acids can possibly involve the action of both dissociated and undissociated forms. Our finding that hydrochloric acid causes significant OM damage at pH 4 shows that the disintegration of the LPS layer can be caused by a fully dissociable acid. The additional OM-disintegrating effect demonstrated here for lactic acid is likely due to the action of undissociated lactic acid molecules; at pH 4 ca. 40% and at pH 3.6 ca. 60% of lactic acid are present in the undissociated form. This conclusion is further supported by our finding that the dissociated potassium lactate at neutral conditions had no permeabilizing activity. Although the addition

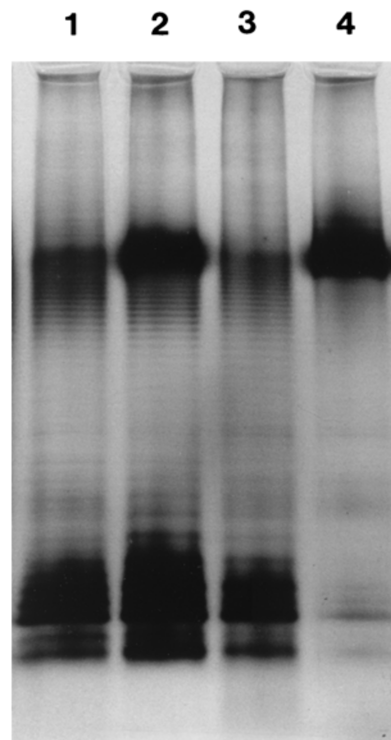


FIG. 1. Silver-stained SDS-polyacrylamide gel (18% acrylamide) of proteinase K-treated cell-free supernatants of serovar Typhimurium SL696 exposed to HCl (pH 3.6) (lane 1), lactic acid (5 mM) (lane 2), or EDTA (1 mM) (lane 3); lane 4 shows the control supernatant. An equal volume of each sample was electrophoresed.

TABLE 3. Liberation of fatty acid-containing material from serovar Typhimurium SL696 by EDTA, lactic acid, and HCl

Fatty acid	Amt (μg) of fatty acid in 8.6 ml of cell-free supernatant after treatment with:			
	None (control)	EDTA (1 mM)	Lactic acid (5 mM, pH 3.5)	HCl (pH 3.5)
$C_{12:0}$ [a]	1.4	2.4	2.8	2.0
$C_{14:0}$ [a]	0.9	4.1	4.7	2.6
$C_{14:0(3\text{-}OH)}$ [a]	0.8	6.1	8.6	4.6
$C_{16:0}$	3.4	6.8	10.7	7.2
$C_{16:1}$	2.3	5.5	8.6	6.0
$C_{18:1}$	2.5	3.1	5.5	3.1
Total	11.3	28.0	40.9	25.5

[a] The percentages of LPS-specific fatty acids for the four treatment groups in columns 2 through 5 are 27, 45, 39, and 36%, respectively.

of $MgCl_2$ to the NPN assay system with 5 mM lactic acid challenge resulted in reduced NPN uptake for *P. aeruginosa* and *E. coli* especially in the presence of KCN, these effects cannot be regarded as indicative of chelation of cations from the OM, since similar effects were also observed with HCl and *E. coli*. A more likely mechanism than chelation would be protonation of anionic components such as carboxyl and phosphate groups and the consequent weakening of molecular interactions between OM components. It is plausible that rather than interacting directly with the acid molecules, $MgCl_2$ stabilizes the OM, making it more resistant to acid challenge. Instead, excess Mg^{2+} with each bacterial species expectedly abolished the NPN uptake induced by EDTA, which is believed to act solely by chelation.

The permeabilizing capacity of lactic acid has a number of important consequences. Above all, lactic acid should be able to potentiate the apparent antimicrobial activity of other components against gram-negative bacteria. In natural situations such as in fermented low-pH products obtained by lactic acid starter culture bacteria, numerous metabolites are present that are too lipophilic or too large to effectively penetrate the intact gram-negative bacterial OM but that could possibly do so in the presence of lactic acid. Beside well-recognized lactic acid bacterial antimicrobial factors such as diacetyl (24), hydrogen peroxide, lactoperoxidase systems, and reuterin (5), a plethora of cryptic antimicrobials acting in synergy with lactic acid could theoretically exist. In fact, culture supernatant of *Lactobacillus plantarum* was recently shown to contain small-molecular-mass substances acting together with lactic acid against the gram-negative target organism *Pantoea agglomerans* (19). There are also indications (3, 4) that high concentrations of lactic acid sensitizes gram-negative bacteria to bacteriocins such as nisin. The sublethal injury caused by lactic acid could play a major role in such sensitizing, along with providing an acidic milieu required for the chemical stability of nisin (6).

Despite the neutral pH conditions of the large intestine, which probably are not favorable for the permeabilizing action of lactic acid in general, it could be assumed that probiotic lactic acid bacterial strains might, however, be beneficial in combating gram-negative pathogens in the large intestine. This could happen through local production of relevant concentrations of lactic acid in microenvironments, with inhibition of harmful gram-negative strains by the combined action of lactic acid and bile salts; the latter possess detergent-like action against which many enteric gram-negative pathogens exhibit resistance (18). Another interesting feature is that lactobacilli and lactic acid have been reported to suppress the gastric pathogen *Helicobacter pylori* (1, 16, 17). *H. pylori* is naturally adapted to an acid environment (9), and it would be of interest to investigate the permeability properties of the OM of this pathogen in response to challenge with different acids.

A gradual increase in acidity can allow induced tolerance to acid to occur (acid habituation), and this will permit the organisms to survive subsequent exposures which could be lethal to nonhabituated cells (2, 20, 21, 27). The effect of such an adaptive response on the permeability properties of gram-negative bacteria should be examined, especially with enteric pathogens such as *E. coli* O157:H7.

ACKNOWLEDGMENTS

We thank Päivi Lepistö and Anna-Liisa Ruskeepää for excellent technical assistance.

This work was supported by the Academy of Finland (project 44163) and by the European Commission (project NISIN[PLUS], FAIR-CT96-1148).

REFERENCES

1. **Aiba, Y., N. Suzuki, A. M. A. Kabir, A. Takagi, and Y. Koga.** 1998. Lactic acid-mediated suppression of *Helicobacter pylori* by the oral administration of *Lactobacillus salivarius* as a probiotic in a gnotobiotic murine model. Am. J. Gastroenterol. **93:**2097–2101.
2. **Baik, H. S., S. Bearson, S. Dunbar, and J. W. Foster.** 1996. The acid tolerance response of *Salmonella typhimurium* provides protection against organic acids. Microbiology **142:**3195–3200.
3. **Cutter, C. N., and G. R. Siragusa.** 1995. Population reductions of gram-negative pathogens following treatments with nisin and chelators under various conditions. J. Food Prot. **58:**977–983.
4. **Cutter, C. N., and G. R. Siragusa.** 1995. Treatments with nisin and chelators to reduce *Salmonella* and *Escherichia coli* on beef. J. Food Prot. **58:**1028–1030.
5. **Daeschel, M. A., and M. H. Penner.** 1992. Hydrogen peroxide, lactoperoxidase systems, and reuterin, p. 155–175. *In* B. Ray and M. Daeschel (ed.), Food preservatives of microbial origin. CRC Press, Boca Raton, Fla.
6. **De Vuyst, L., and E. J. Vandamme.** 1994. Nisin, a lantibiotic produced by *Lactococcus lactis* subsp. *lactis*: properties, biosynthesis, fermentation and application, p. 151–221. *In* L. De Vuyst and E. J. Vandamme (ed.), Bacteriocins of lactic acid bacteria. Blackie A&P, Glasgow, United Kingdom.
7. **Dickson, J. S., and M. E. Anderson.** 1992. Microbiological decontamination of food animal carcasses by washing and sanitising systems: a review. J. Food Prot. **55:**133–140.
8. **Doores, S.** 1993. Organic acids, p. 95–136. *In* P. M. Davidson and A. L. Branen (ed.), Antimicrobials in foods. Marcel Dekker, Inc., New York, N.Y.
9. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin. Microbiol. Rev. **10:**720–741.
10. **Greer, G. G., and B. D. Dilts.** 1995. Lactic acid inhibition of the growth of spoilage bacteria and cold tolerant pathogens on pork. Int. J. Food Microbiol. **25:**141–151.
11. **Helander, I. M., P. H. Mäkelä, O. Westphal, and E. T. Rietschel.** 1996. Lipopolysaccharides, p. 462–471. *In* R. A. Meyers (ed.), Encyclopedia of molecular biology and molecular medicine, vol. 3. VCH, Weinheim, Germany.
12. **Helander, I. M., H.-L. Alakomi, K. Latva-Kala, and P. Koski.** 1997. Polyethyleneimine is an effective permeabilizer of gram-negative bacteria. Microbiology **143:**3193–3199.
13. **Helander, I. M., A. von Wright, and T. Mattila-Sandholm.** 1997. Potential of lactic acid bacteria and novel antimicrobials against gram-negative bacteria. Trends Food Sci. Technol. **8:**146–150.
14. **Helander, I. M., H.-L. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E. J. Smid, L. G. M. Gorris, and A. von Wright.** 1998. Characterization of the action of selected essential oil components on gram-negative bacteria. J. Agric. Food Chem. **46:**3590–3595.
15. **Helander, I. M., and T. Mattila-Sandholm.** 2000. Fluorometric assessment of gram-negative bacterial permeabilization. J. Appl. Microbiol. **88:**213–219.
16. **Kabir, A. M. A., Y. Aiba, A. Takagi, S. Kamiya, T. Miwa, and Y. Koga.** 1997. Prevention of *Helicobacter pylori* infection by lactobacilli in a gnotobiotic murine model. Gut **41:**49–55.
17. **Midolo, P. D., J. R. Lambert, R. Hull, F. Luo, and M. L. Grayson.** 1995. *In vitro* inhibition of *Helicobacter pylori* NCTC 11637 by organic acids and lactic acid bacteria. J. Appl. Bacteriol. **79:**475–479.
18. **Nikaido, H.** 1996. Outer membrane, p. 29–47. *In* F. C. Neidhardt et al. (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. American Society for Microbiology, Washington, D.C.
19. **Niku-Paavola, M.-L., A. Laitila, T. Mattila-Sandholm, and A. Haikara.** 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. J. Appl. Microbiol. **86:**29–35.

20. **Nojoumi, S. A., D. G. Smith, and R. J. Rowbury.** 1995. Tolerance to acid in pH 5.0-grown organisms of potentially pathogenic gram-negative bacteria. Lett. Appl. Microbiol. **21:**359–363.
21. **Park, Y.-K., B. Bearson, S. H. Bang, I. S. Bang, and J. W. Foster.** 1996. Internal pH crisis, lysine decarboxylase and the acid tolerance response of *Salmonella typhimurium*. Mol. Microbiol. **20:**605–611.
22. **Podolak, R. K., J. F. Zayas, C. L. Kastner, and D. Y. C. Fung.** 1996. Inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7 on beef by application of organic acids. J. Food Prot. **59:**370–373.
23. **Przybylski, K. S., and L. D. Witter.** 1979. Injury and recovery of *Escherichia coli* after sublethal acidification. Appl. Environ. Microbiol. **37:**261–265.
24. **Ray, B.** 1992. Diacetyl of lactic acid bacteria as a food biopreservative, p. 137–153. *In* B. Ray and M. Daeschel (ed.), Food preservatives of microbial origin. CRC Press, Boca Raton, Fla.
25. **Ray, B., and W. E. Sandine.** 1992. Acetic, propionic, and lactic acids of starter culture bacteria as biopreservatives, p. 103–136. *In* B. Ray and M. Daeschel (ed.), Food preservatives of microbial origin. CRC Press, Boca Raton, Fla.
26. **Roth, L. A., and D. Keenan.** 1971. Acid injury in *Escherichia coli*. Can. J. Microbiol. **17:**1005–1008.
27. **Ryu, J.-H., Y. Deng, and L. Beuchat.** 1999. Behavior of acid-adapted and unadapted *Escherichia coli* O157:H7 when exposed to reduced pH achieved with various organic acids. J. Food Prot. **62:**451–455.
28. **Salminen, S., M. A. Deighton, Y. Benno, and S. L. Gorbach.** 1998. Lactic acid bacteria in health and disease, p. 211–253. *In* S. Salminen and A. von Wright (ed.), Lactic acid bacteria: microbiology and functional aspects. Marcel Dekker, Inc., New York, N.Y.
29. **Smulders, F. J. M., and G. G. Greer.** 1998. Integrating microbial decontamination with organic acids in HACCP programmes for muscle foods: prospects and controversies. Int. J. Food Microbiol. **44:**149–169.
30. **Träuble, H., and P. Overath.** 1973. The structure of *Escherichia coli* membranes studied by fluorescence measurements of lipid phase transition. Biochim. Biophys. Acta **307:**491–512.
31. **Vaara, M.** 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev. **56:**395–411.
32. **van Netten, P., J. H. J. Huis In't Veld, and D. A. A. Mossel.** 1994. An *in-vitro* meat model to assess the immediate bactericidal effect of lactic acid decontamination of meat. J. Appl. Bacteriol. **76:**49–54.
33. **van Netten, P., D. A. A. Mossel, and J. H. J. Huis In't Veld.** 1995. Lactic acid decontamination of fresh pork carcasses: a pilot plant study. Int. J. Food Microbiol. **21:**1–15.
34. **Wilkinson, R. G., P. Gemski, Jr., and B. A. D. Stocker.** 1972. Non-smooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. J. Gen. Microbiol. **70:**527–554.
35. **Zähringer, U., B. Lindner, and E. T. Rietschel.** 1994. Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. Adv. Carbohydr. Chem. Biochem. **50:**211–276.

PAPER II

Effect of EDTA on *Salmonella enterica* serovar Typhimurium involves a component not assignable to lipopolysaccharide release

In: Microbiology 2003. Vol. 149, pp. 2015–2021.
Reprinted with permission from the publisher.

Microbiology (2003), **149**, 2015–2021 **DOI** 10.1099/mic.0.26312-0

Effect of EDTA on *Salmonella enterica* serovar Typhimurium involves a component not assignable to lipopolysaccharide release

H.-L. Alakomi,[1] M. Saarela[1] and I. M. Helander[1,2]

[1]VTT Biotechnology, PO Box 1500, FIN-02044 VTT, Espoo, Finland

[2]Department of Applied Chemistry and Microbiology, Division of Microbiology, University of Helsinki, PO Box 56, FIN-00014 University of Helsinki, Finland

Correspondence
H.-L. Alakomi
Hanna-Leena.Alakomi@vtt.fi

Received 20 February 2003
Revised 16 April 2003
Accepted 28 April 2003

The effect of EDTA on *Salmonella enterica* serovar Typhimurium was studied in different growth phases with cells grown with or without Ca^{2+} and Mg^{2+} supplementation. EDTA affected the outer membrane much more strongly in the early exponential phase than in the mid- or late exponential phase, as indicated by uptake of 1-*N*-phenylnaphthylamine (a nonpolar hydrophobic probe, M_r 219), and detergent (SDS) susceptibility. This effect was, however, not paralleled by LPS release (determined by measuring LPS-specific fatty acids or ^{14}C-labelled LPS in cell-free supernatants, per a standardized cell density), which remained unchanged as a function of the growth curve. The conclusion from these results is that in the early exponential phase the effect of EDTA in *S. enterica* involves a component that is independent of LPS release.

INTRODUCTION

The Gram-negative outer membrane (OM) functions as a barrier for many external agents, protecting the cells from the detergent action of bile salts and degradation by digestive enzymes (Nikaido, 1989, 1996; Vaara, 1999). The effect is mainly due to the presence and features of lipopolysaccharide (LPS) molecules in the outer leaflet of the membrane, resulting in many Gram-negative bacteria having an inherent resistance to hydrophobic antibiotics (e.g. macrolides, novobiocins, rifamycins, actinomycin D), detergents (e.g. bile salts, SDS, Triton X-100) and hydrophobic dyes (e.g. eosin, methylene blue, brilliant green, acridine dyes) (Vaara, 1999). In *Escherichia coli* and *Salmonella enterica*, the LPS molecules consist of (1) a hydrophobic membrane anchor lipid part, termed lipid A, (2) a core oligosaccharide with multiple phosphoryl substituents, and (3) a structurally diverse polymer composed of oligosaccharide repeats, termed the O antigen, protruding outwards and providing the cell with a hydrophilic surface (for reviews see Helander *et al.*, 1996; Raetz & Whitfield, 2002; Yethon & Whitfield, 2001a). In particular, negatively charged residues in the inner (lipid A-proximal) region of the LPS core oligosaccharide are critical to membrane integrity. These negative charges, provided by residues of 3-deoxy-D-*manno*-octulosonic acid (Kdo) and phosphate, allow neighbouring LPS molecules to be cross-linked by divalent cations (Mg^{2+}, Ca^{2+}), structurally reinforcing the OM (Nikaido, 1996). Since the initial research by Leive (1965) it has been known that chelating agents such as EDTA destabilize the OM of Gram-negative bacteria by sequestering the stabilizing divalent cations. Such destabilization leads to the release of substantial proportions, up to 40 %, of LPS (Leive, 1965, 1974; Hukari *et al.*, 1986; Alakomi *et al.*, 2000), whereby EDTA-treated bacteria become susceptible to agents that normally do not penetrate the OM and, as a consequence, do not affect the bacteria, as summarized by Vaara (1992, 1999). This phenomenon is often referred to as permeabilization.

The permeabilizing action of EDTA is usually considered to result from LPS release and from consequent perturbations of OM structure and function (Vaara, 1992), but there are still unexplained features of the mechanism. It is not known, for instance, why only a certain proportion of LPS is released. It was shown by Hukari *et al.* (1986) that the macromolecular quality (LPS chain length distribution) of EDTA-released LPS vs cell-bound LPS was identical. It can, however, be postulated that the releasable fraction differs from the non-releasable one in some structural aspect that is related to the stabilizing effect of divalent cations, perhaps at the level of anionic groups in the core oligosaccaharide and lipid A.

In our studies concerning permeabilization of Gram-negative bacteria we have utilized several methods to unravel the mechanisms underlying these phenomena. In addition to measuring LPS release, the sensitization of bacteria to lysozyme and detergents is used to measure alterations in OM function (Helander *et al.*, 1997a, 1998;

Abbreviations: KDO, 3-deoxy-D-*manno*-octulosonic acid; NPN, 1-*N*-phenylnaphthylamine; OM, outer membrane.

Alakomi *et al.*, 2000). Yet another method is the application of a nonpolar hydrophobic probe, 1-*N*-phenylnaphthylamine (NPN). NPN fluoresces strongly in glycerophospholipid environments, but only weakly in aqueous environments (Träuble & Overath, 1973). This is utilized in fluorometric studies of the permeability of OMs, as increased fluorescence in suspensions of Gram-negative bacteria can be used as a measure of functional changes of the OM (Loh *et al.*, 1984; Helander & Mattila-Sandholm, 2000).

By applying several independent methods to the study of EDTA-induced permeabilization in *S. enterica* we have noticed that mere LPS release does not account for OM alterations. The results leading to this conclusion are presented in this paper. These results have been in part presented in a poster by H.-L. Alakomi & I. M. Helander at the 6th Conference of the International Endotoxin Society, Paris, 24–27 August 2000.

METHODS

Chemicals. Triton X-100 was from BDH; chicken egg white lysozyme (EC 3.2.1.17), HEPES, n-heptadecanoic acid methyl ester, 1-*N*-phenylnaphthylamine (NPN) and SDS were from Sigma-Aldrich; D-[1-^{14}C]galactose (specific activity 49·4 μCi mmol^{-1}, 1829 kBq mmol^{-1}) was from Amersham Pharmacia Biotech; and EDTA was from Riedel-de-Haen. A stock solution of NPN (0·5 M) was prepared in acetone and diluted to 40 μM into 5 mM HEPES (pH 7·2) for the fluorometric assays.

Test strain and growth conditions. *Salmonella enterica* serovar Typhimurium VTT E-95582^{T} (ATCC 13311^{T}) cells were grown in Luria–Bertani broth (LB) at 37 °C as described previously (Helander *et al.*, 1997a). Experiments in LB were performed with either 2 mM $CaCl_2$ or 2 mM $MgCl_2$ addition or in LB without added salts. The amounts of calcium and magnesium in the LB without added salts were 0·26 mM and 0·23 mM, respectively (determined by atomic absorption spectrometry: AAS). The effect of the growth phase on EDTA sensitivity was studied by using cells harvested in different growth phases: early exponential (OD_{630} 0·20 ± 0·02), mid-exponential (OD_{630} 0·50 ± 0·02) and late exponential (OD_{630} 0·70 ± 0·02). After harvesting, the cells were washed in the appropriate buffer and resuspended to a standardized optical density. Efficacy of washing treatment to remove the excess salts was verified by AAS. No significant differences in the amounts of calcium and magnesium in the washed cell suspensions were observed (< 0·04 mM and 0·04 mM respectively). Further details of cell treatments are given below under various experimental settings.

Permeability assays. Two methods were utilized to determine permeability properties of the OM: (i) NPN uptake and (ii) sensitization to bacteriolysis induced by detergents or lysozyme.

(i) NPN uptake by bacterial suspensions was measured using black fluorotitre plates (cat. no. 9502 867, LabSystems) and the automated fluorometer Fluoroskan Ascent FL (LabSystems) as described earlier (Alakomi *et al.*, 2000; Helander & Mattila-Sandholm, 2000). Briefly, cells grown to different growth phases were deposited by centrifugation at room temperature for 10 min at 1000 ***g***, washed with 5 mM HEPES buffer (pH 7·2), and the suspension's optical density was adjusted to OD_{630} 0·5 ± 0·02 with the same buffer. After centrifugation as above the cells were suspended into 0·5 vol. 5 mM HEPES buffer. Aliquots (100 μl) of this cell suspension were pipetted into fluoroplate wells containing NPN (10 μM), and as test substances either EDTA (1·0 and 0·1 mM) or HEPES buffer (control) to make up a total volume of 200 μl. If desired, $MgCl_2$ was added to the cell suspension before addition of NPN. Fluorescence was monitored within 3 min from four parallel wells per sample (excitation, 355 nm, half bandwidth 38 ± 3 nm; emission, 402 nm, half bandwidth 50 ± 5 nm). Each assay was performed at least three times.

(ii) Sensitization of target cells to the lytic action of lysozyme and the detergents SDS and Triton X-100 by EDTA was investigated according to the method described in detail by Helander *et al.* (1997a). Briefly, bacteria at standardized OD_{630} 0·5 ± 0·02 were subjected to treatments with EDTA (0·1 and 1·0 mM) for 10 min at room temperature and added to microtitre plate wells which already contained either lysozyme (10 μg ml^{-1}), SDS (0·05 and 0·1 %), Triton X-100 (0·1 and 1·0 %) or buffer only. Turbidity of the cell suspensions was then monitored with the Multiskan MCC/340 spectrophotometer (LabSystems).

Results from the permeability assays were analysed statistically using two-tailed unpaired Student's *t*-test to determine differences.

LPS release. EDTA-induced LPS/lipid release was studied in different growth phases and measured by two methods.

(i) Release of LPS and glycerophospholipids. The release of lipid components, including LPS, was assayed by fatty acid analysis (gas chromatography of fatty acid methyl esters) of cell-free supernatants after treatment of the bacterial suspensions with EDTA. Cells in different growth phases were collected by centrifugation, washed with 10 mM Tris/HCl (pH 7·2) and resuspended to OD_{630} 0·5 ± 0·02 in the same buffer containing either 0·1 or 1·0 mM EDTA (total volume, 10 ml). The control sample was suspended in buffer only. After a 10 min incubation at 37 °C with shaking (150 r.p.m.) the samples were distributed into Eppendorf tubes and centrifuged (13 000 ***g***) in an Eppendorf microfuge for 1 min at room temperature. A total of 9·1 ml of cell-free supernatants per sample was collected, freeze-dried and processed for fatty acid analysis by saponification and methylation as described by Helander *et al.* (1997a).

(ii) Radiolabelling of lipopolysaccharide, release of [^{14}C]Gal-LPS. Smooth *S. enterica* Typhimurium E- 95582^{T} cells were grown in LB at 37 °C with shaking (200 r.p.m.) to the desired growth phase and supplemented with [^{14}C]galactose (0·1 μCi ml^{-1}) in order to label their LPS (Hukari *et al.*, 1986). Labelling with [^{14}C]galactose was performed for 5 min at 37 °C with shaking (200 r.p.m.). A 1 ml aliquot was then removed for checking the level of incorporation of radiolabel (Wallac 1410 Liquid Scintillation Counter, Pharmacia). The remaining cells were collected by centrifugation (1000 ***g***, 10 min, 25 °C), and washed with 10 mM Tris/HCl buffer (pH 7·2) at room temperature. After centrifugation the cells were resuspended in the same buffer to OD_{630} 0·5 ± 0·02, divided into three portions and supplemented with either 1·0 or 0·1 mM EDTA; buffer was used as control. After adding the test substance, the assay suspensions were incubated at 37 °C for 10 min with shaking (100 r.p.m.). Samples (2 × 20 μl) were taken for radioactivity measurements and remaining cells were centrifuged twice (1500 ***g***) at room temperature. After centrifugation, samples from the cell-free supernatants were taken for radioactivity measurements. The amount of radioactivity in the cell-free supernatant was taken as the measure of liberated LPS and the percentage value for LPS release was calculated by comparison to the radioactivity of a similar volume of untreated and uncentrifuged bacterial suspension. Whereas it is known that the buffer used, Tris, a bulky primary amine, alone at high concentrations (50 mM or higher, pH 7·2) can cause release of some LPS and to make bacterial cells somewhat more sensitive to various agents (Vaara, 1992), its concentration in these experiments was only 10 mM, and we observed no additional sensitivity due to the buffer.

RESULTS

NPN uptake by cells in different growth phases

S. enterica serovar Typhimurium E-95582^T grown in LB showed a typical growth curve with lag and exponential phases. The effect of the growth phase on EDTA sensitivity was studied by using cells harvested in different growth phases: early exponential (OD_{630} 0·20±0·02), mid-exponential (OD_{630} 0·50±0·02) and late exponential (OD_{630} 0·70±0·02). With 2 mM $CaCl_2$ or 2 mM $MgCl_2$ supplementation no significant difference in the shape of the growth curve was observed (data not shown).

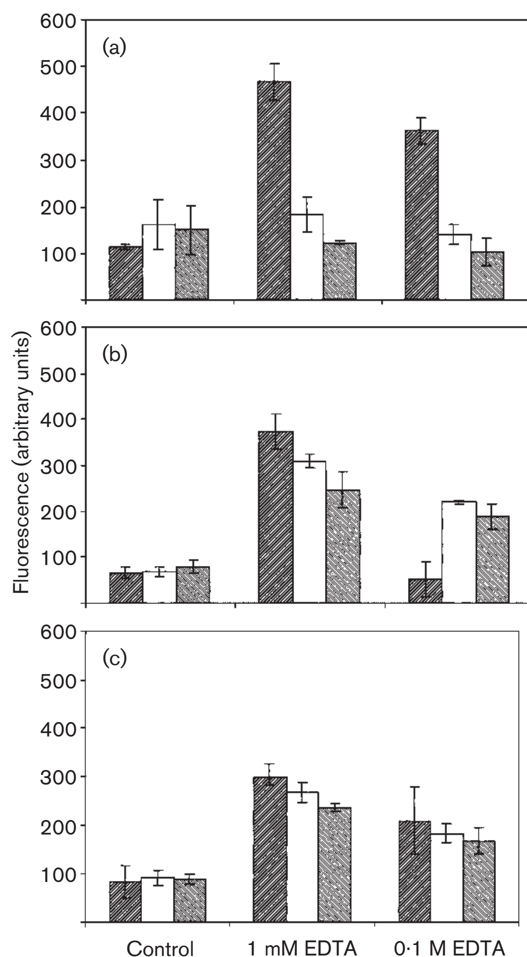


Fig. 1. NPN uptake in suspensions of *S. enterica* serovar Typhimurium E-95582^T cells harvested at different growth phases: early exponential (hatched columns), mid-exponential (white columns) and late exponential (shaded columns). Cells were cultivated in LB (a), or in LB supplemented with 2 mM $CaCl_2$ (b) or with 2 mM $MgCl_2$ (c). Upon treatment with 1 mM EDTA the cells grown in LB with or without salt additions showed increased NPN uptake. This phenomenon was significant in EDTA-treated early exponential phase versus late exponential phase cells ($P<0·001$, panel a; $P<0·01$, panel b).

Fig. 1 shows results obtained in the NPN assay with cells grown in LB with salts and without salt additions. Fig. 1(a) shows that the NPN uptake by cells grown in LB did not differ significantly at different growth phases (fluorescence levels of control cells from 100 to 200 units). Upon treatment with EDTA, however, the cells showed increased NPN uptake, but this phenomenon was significant at the early exponential phase of growth only. Addition of 2 mM $CaCl_2$ to the growth medium stabilized the OM, as indicated by the lower NPN uptake of the control cells (Fig. 1b, fluorescence levels of less than 100 units). In general, supplementation of cells with Ca^{2+} during growth considerably increased their NPN uptake induced by EDTA, especially in the mid- and late exponential phases. Accordingly, significant increases in NPN uptake by 1·0 mM EDTA were observed at all growth phases, cells in the earlier phases again exhibiting higher uptakes. An EDTA concentration of 0·1 mM was obviously not sufficient to destabilize and increase the permeability of the OM in early exponential cells (OD_{630} 0·2) grown in LB supplemented with 2 mM $CaCl_2$, indicating the presence of massive ionic interactions within the OM (Fig. 1b). In the presence of 2 mM added Mg^{2+} the results with 1 mM EDTA (Fig. 1c) were similar but not as high as with Ca^{2+} addition. However, the presence of 2 mM Mg^{2+} during cultivation was sufficient to destabilize and increase the permeability of the target cells by 0·1 mM EDTA already in the early growth phase. Addition of 1 mM $MgCl_2$ to the buffer used in the NPN assay abolished the permeabilizing activity of EDTA (data not shown) and the NPN uptake of target cells was at the same level as in the corresponding control cells. The conclusion from these experiments was that the growth phase has a profound effect on the bacterium's sensitivity to EDTA as assayed by NPN uptake, with early exponential phase cells exhibiting particularly high sensitivity.

Sensitization to lytic agents

Increased permeability of the OM is also manifested as an increased susceptibility to the bacteriolytic action of detergents and to the cell-wall-degrading action of lysozyme (Vaara, 1992). To further investigate the sensitivity of *S. enterica* Typhimurium cells in different growth phases we therefore tested the effect of EDTA on the susceptibility of cells to lysozyme- and detergent-induced cell lysis. The results are compiled in Tables 1 and 2. Table 1 shows that significant lysis by Triton X-100 did not occur in control cells, whereas SDS (anionic detergent probe) itself somewhat lysed the control cells. Treatment with EDTA, however, sensitized LB-grown cells to SDS (0·05 and 0·1 %). This sensitization was quantitatively similar in each growth phase. Table 2 shows that early exponential phase cells grown in LB supplemented with Ca^{2+} and pretreated with 0·1 mM EDTA were more resistant to lysis by 0·05 % SDS than cells grown without added Ca^{2+} (Table 1), a result in accordance with the similar finding in the NPN uptake experiment. These cells were equally sensitized to the action of 0·1 % SDS/1 mM EDTA in early and late exponential

Table 1. Sensitization of *S. enterica* serovar Typhimurium E-95582[T], grown in LB, to detergent-induced bacteriolysis after pretreatments with EDTA

Lytic agent (concn)	Relative turbidity (%) at 4 min								
	Early exponential cells (OD_{630} 0·2±0·02)			Mid-exponential cells (OD_{630} 0·5±0·02)			Late exponential cells (OD_{630} 0·7±0·02)		
	Control	0·1 mM EDTA	1 mM EDTA	Control	0·1 mM EDTA	1 mM EDTA	Control	0·1 mM EDTA	1 mM EDTA
Lysozyme (10 μg ml^{-1})	106±3	102±3	101±2	106±1	97±2	97±2	102±3	95±2	96±3
Triton X-100 (0·1%)	109±3	98±2	95±2	109±3	100±3	99±1	106±2	103±2	101±2
Triton X-100 (1%)	105±4	91±2	90±2	104±3	94±3	94±2	102±2	98±2	96±1
SDS (0·05%)	88±7	36±1*	37±2*	91±4	30±2*	29±4*	96±1	41±4*	41±3*
SDS (0·1%)	63±5	19±2*	20±2*	75±10	18±2*	19±1*	85±5	21±1*	21±1*

*$P<0·001$ compared to the control value.

phases. Notably, the presence of 2 mM Ca^{2+} during cultivation rendered the early exponential phase cells sensitive to lysis by 1% Triton X-100/1 mM EDTA. In conclusion, the results of the cell lysis experiments do not indicate major differences in functional properties of *S. enterica* Typhimurium OMs as a function of the growth phase.

Release of LPS

Since EDTA seemed to weaken the OM markedly in the early exponential phase and since it is known to destabilize OMs by liberating LPS, we studied the amount of LPS and lipid material released by EDTA at different growth phases by analysing cell-free supernatants derived from cell suspensions for lipid components. Table 3 shows that EDTA liberated lipid material, including LPS, as indicated by the LPS-specific fatty acids C12:0, C14:0, C14:0(3OH), and glycerophospholipid, as indicated by fatty acids C16:0, C16:1, C18:1. Ca^{2+} supplementation during growth increased the total amount of liberated fatty acids. However, no significant difference was found in the amounts of LPS liberated by EDTA from cells grown to the early or late exponential phase. A similar result was obtained from experiments involving specific labelling of LPS ([^{14}C]Gal-LPS) and analysis of EDTA-releasable [^{14}C]Gal-LPS at three different growth phases (Table 4). The conclusion thus is that LPS release by EDTA is not dependent on growth phase in *S. enterica* Typhimurium E-95582[T].

DISCUSSION

The results presented above demonstrate that the effect of EDTA on *S. enterica* cells involves a component that is independent of LPS release, the classical explanation for the mechanism of EDTA-induced permeabilization of Gram-negative bacterial OMs. This component was indicated by the significantly higher NPN uptake observed in early exponential phase cells as compared to late exponential phase cells. Release of LPS, as determined by measurement in cell-free supernatants of either LPS-specific fatty acids or radiolabelled LPS, per a standardized cell density, remained virtually unchanged along the growth curve. Another method to test permeabilization, i.e. sensitization to lytic agents, yielded mostly results paralleling those of the LPS release measurements. It can thus be concluded that in addition to its LPS-releasing mechanism, EDTA in the early exponential

Table 2. Sensitization of *S. enterica* serovar Typhimurium E-95582[T], grown in LB supplemented with 2 mM $CaCl_2$, to detergent-induced bacteriolysis after pretreatments with EDTA

Lytic agent (concn)	Relative turbidity (%) at 4 min					
	Early exponential cells (OD_{630} 0·2±0·02)			Late exponential cells (OD_{630} 0·7±0·02)		
	Control	0·1 mM EDTA	1 mM EDTA	Control	0·1 mM EDTA	1 mM EDTA
Lysozyme (10 μg ml^{-1})	110±3	100±1	100±3	104±2	98±1	97±2
Triton X-100 (0·1%)	111±3	110±2	66±7*	111±1	96±3	89±6
Triton X-100 (1%)	110±5	103±2	61±7*	107±1	90±1	85±6
SDS (0·05%)	99±3	60±7*	28±5*	96±1	38±1*	39±1*
SDS (0·1%)	63±17	27±5	16±3*	82±2	20±1*	20±1*

*$P<0·001$ compared to the control value.

Table 3. Liberation of lipid material from *S. enterica* serovar Typhimurium E-95582[T]

Fatty acid	Amount of fatty acid in 9·1 ml cell-free supernatant after treatment of cells with:					
	Control		1 mM EDTA		0·1 mM EDTA	
	LB	LB+$CaCl_2$	LB	LB+$CaCl_2$	LB	LB+$CaCl_2$
Early exponential phase cells (OD_{630} 0·2)						
12:0†	0·7	1·1	1·9	3·0	2·2	2·8
14:0†	0·4	0·4	2·4	4·4	2·2	3·5
16:0	1·8	2·0	6·5	11·2	5·8	7·3
18:0	0·3	0·6	0·5	1·0	0·5	0·5
17:0 cyc	0·0	0·0	0·0	0·9	0·0	0·5
16:1	0·9	1·2	4·5	7·5	3·6	5·4
18:1	1·2	1·1	3·7	6·5	3·7	4·5
14:0 3 (OH)†	0·0	0·0	4·9	7·1	3·3	8·5
Sum of all fatty acids	5·4	6·4	24·5	41·6	21·2	33·0
Percentage of LPS-specific† fatty acids	20	23	38	35	36	45
Late exponential phase cells (OD_{630} 0·7)						
12:0†	1·1	0·6	1·8	2·7	1·6	2·7
14:0†	0·4	0·6	2·1	3·7	1·9	3·1
16:0	2·3	3·0	6·2	11·2	5·5	10·2
18:0	0·0	1·1	0·0	0·7	0·0	0·8
17:0 cyc	0·0	0·0	0·7	0·6	0·7	0·4
16:1	1·1	1·4	3·4	6·4	3·0	5·4
18:1	1·6	1·9	3·9	8·6	3·9	7·0
14:0 3 (OH)†	1·1	0·8	4·3	9·2	3·5	5·0
Sum of all fatty acids	7·5	9·5	22·4	43·2	20·2	34·7
Percentage of LPS-specific† fatty acids	35	21	37	36	35	31

phase of growth acts upon cells by another mechanism that does not involve LPS release.

The mechanism underlying the sensitization to EDTA in the early exponential growth phase remains unknown at present, but its existence opens up interesting possibilities concerning stability-affecting properties of OM components. Since NPN fluorescence is associated with the presence of this hydrophobic probe in a glycerophospholipid environment (Träuble & Overath, 1973), it is evident that early exponential phase cells are very susceptible to the effect of EDTA in allowing access of NPN to glycerophospholipids either directly on the OM surface or via the periplasm. If differences in the stability properties of OM components are responsible for the effects described, there should be demonstrable differences in the structure of OM components as a function of the growth phase. Such differences could be expected to be found in the fine structure of LPS, especially in the degree of substitution of phosphate groups of lipid A and the core oligosaccharide. One could postulate, in addition to the EDTA-releasable fraction, the presence of a population of LPS whose interactions with neighbouring components are disturbed by EDTA to an extent that does not, however, result in LPS release. In order to prove this, the LPS obtained from distinct points of the growth phase should be scrutinized for phosphate substituents known to be critical for OM integrity (Helander *et al.*, 1997b; Raetz & Whitfield, 2002; Yethon *et al.*, 1998; Yethon & Whitfield, 2001b). In addition, LPS fractions which are releasable and non-releasable by EDTA should be studied for similar fine

Table 4. EDTA-induced [^{14}C]LPS release from *S. enterica* serovar Typhimurium E-95582[T] in different growth phases

Addition to assay buffer	Release of LPS (%)					
	Early exponential cells (OD_{630} 0·2±0·02)		Mid-exponential cells (OD_{630} 0·5±0·02)		Late exponential cells (OD_{630} 0·7±0·02)	
	LB	LB+2 mM $CaCl_2$	LB	LB+2 mM $CaCl_2$	LB	LB+2 mM $CaCl_2$
Control	5±2	4±1	12±4	8±3	18±9	16±3
EDTA (0·1 mM)	20±3	33±7	23±9	45±0	23±2	39±6
EDTA (1 mM)	16±2	29±4	21±3	37±7	24±1	37±9

structures. In this context it is interesting to note the recent report of Kanipest *et al.* (2001), who demonstrated in *E. coli* that the critical parameter determining the presence or absence of phosphoethanolamine was the $CaCl_2$ concentration in the medium. They observed a novel $CaCl_2$-induced enzyme that modifies the outer Kdo moiety of *E. coli* LPS with a phosphoethanolamine group in the presence of 5–50 mM $CaCl_2$. Such a modification would increase the average resistance of LPS molecules to the releasing action of EDTA, as molecules capped with phosphoethanolamine at Kdo are less prone to be stabilized by divalent cations. Our finding that the addition of Ca^{2+} ions to the growth medium in some cases stabilized the OM is in agreement with this. It is equally true, however, that similar addition of Ca^{2+} can stabilize the OM, as already suggested by Leive (1974); in our experiments this was observed in late exponential phase cells by NPN uptake.

Finch & Brown (1975) observed in *Pseudomonas aeruginosa* with low growth rates increased sensitivity to EDTA (cell lysis) when cells were grown either under carbon limitation or in Ca^{2+}-enriched medium. Furthermore, increased resistance to EDTA in *P. aeruginosa* was reported when cells were grown in Mg^{2+}-limited medium. According to Finch & Brown (1975) the removal of cations from the cell membrane is due to the greater affinity of the cations for EDTA than for cell membrane components. In addition, the higher stability constant for EDTA interaction with Ca^{2+} than with Mg^{2+} (10·7 and 8·7, respectively) also influences the activity of EDTA in the growth medium.

Studies of Kotra *et al.* (2000) employing atomic force microscopy demonstrated that the OM surface in *E. coli* is not uniform; i.e. LPS molecules form distinct patches with depressions in between. Furthermore, a non-uniform distribution of metal ions in the OM was implied, giving rise to local variations in the interactions between OM components. Our findings are in agreement with this view.

Another mechanism could be that efflux pumps that remove substances such as NPN from the periplasm are functionally impaired by EDTA, and that they are present in larger numbers in the early exponential phase of growth as compared to the late exponential phase. In this case the phenomenon is not one of permeabilization but rather a more indirect one.

Finally, our results demonstrate that NPN uptake assays should be carried out with cells that have been cultivated in standardized conditions, especially with respect to their growth phase.

ACKNOWLEDGEMENTS

We thank Päivi Lepistö and Anna-Liisa Ruskeepää for excellent technical assistance. The authors are grateful to Helena Liukkonen-Lilja for the AAS measurements and Kyösti Latva-Kala for the GC measurements. This work was financially supported by the Academy of Finland (Project 44163).

REFERENCES

Alakomi, H.-L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K. & Helander, I. M. (2000). Lactic acid permeabilizes gram-negative bacteria by distrupting the outer membrane. *Appl Environ Microbiol* **66**, 2001–2005.

Finch, J. E. & Brown, R. W. (1975). The influence of nutrient limitation in a chemostat on the sensitivity of *Pseudomonas aeruginosa* to polymyxin and to EDTA. *J Antimicrob Chemother* **1**, 379–386.

Helander, I. M. & Mattila-Sandholm, T. (2000). Fluorometric assessment of Gram-negative bacterial permeabilization. *J Appl Microbiol* **88**, 213–219.

Helander, I. M., Mäkelä, P. H., Westphal, O. & Rietschel, E. T. (1996). Lipopolysaccharides. In *Encyclopedia of Molecular Biology and Molecular Medicine*, vol. 3, pp. 462–471. Edited by R. A. Meyers. Weinheim: VCH.

Helander, I. M., Alakomi, H.-L., Latva-Kala, K. & Koski, P. (1997a). Polyethyleneimine is an effective permeabilizer of gram-negative bacteria. *Microbiology* **143**, 3193–3199.

Helander, I. M., Kilpeläinen, I. & Vaara, M. (1997b). Phosphate groups in lipopolysaccharides of *Salmonella typhimurium rfaP* mutants. *FEBS Lett* **409**, 457–460.

Helander, I. M., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Gorris, L. G. M. & von Wright, A. (1998). Characterization of the action of selected essential oil components on gram-negative bacteria. *J Agric Food Chem* **46**, 3590–3595.

Hukari, R., Helander, I. M. & Vaara, M. (1986). Chain length heterogeneity of lipopolysaccharide released from *Salmonella typhimurium* by ethylenediaminetetraacetic acid or polycations. *Eur J Biochem* **154**, 673–676.

Kanipest, M. I., Lin, S., Cotters, R. J. & Raetz, C. R. H. (2001). Ca^{2+}-induced phosphoethanolamine transfer to the outer 3-deoxy-D-*manno*-octulosonic acid moiety of *Escherichia coli* lipopolysaccharide. *J Biol Chem* **276**, 1156–1163.

Kotra, L. P., Amro, N. A., Liu, G.-Y. & Mobashery, S. (2000). Visualizing bacteria at high resolution. *ASM News* **66**, 675–681.

Leive, L. (1965). Release of lipopolysaccharide by EDTA treatment of *E. coli*. *Biochem Biophys Res Commun* **21**, 290–296.

Leive, L. (1974). The barrier function of gram-negative envelope. *Ann N Y Acad Sci* **235**, 109–127.

Loh, B., Grant, C. & Hancock, R. E. W. (1984). Use of the fluorescent probe 1-*N*-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **26**, 546–551.

Nikaido, H. (1989). Outer membrane barrier as a mechanisms of antimicrobial resistance. *Antimicrob Agents Chemother* **33**, 1831–1836.

Nikaido, H. (1996). Outer membrane. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, vol. 1, pp. 29–47. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.

Raetz, C. R. H. & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**, 635–700.

Träuble, H. & Overath, P. (1973). The structure of *Escherichia coli* membranes studied by fluorescence measurements of lipid phase transition. *Biochim Biophys Acta* **307**, 491–512.

Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiol Rev* **56**, 395–411.

Vaara, M. (1999). Lipopolysaccharide and the permeability of the bacterial outer membrane. In *Endotoxin in Health and Disease*,

pp. 31–38. Edited by H. Brade, S. M. Opal, S. N. Vogel & D. C. Morrison. New York & Basel: Marcel Dekker.

Yethon, J. A. & Whitfield, C. (2001a). Lipopolysaccharide as a target for the development of novel therapeutics in gram-negative bacteria. *Curr Drug Targets Infect Disord* **1**, 91–106.

Yethon, J. A. & Whitfield, C. (2001b). Purification and characterization of WaaP from *Escherichia coli*, a lipopolysaccharide kinase essential for outer membrane stability. *J Biol Chem* **276**, 5498–5504.

Yethon, J. A., Heinrichs, D. E., Monteiro, M. A., Perry, M. B. & Whitfield, C. (1998). Involvement of WaaY, WaaQ, and WaaP in the modification of *Escherichia coli* lipopolysaccharide and their role in the formation of a stable outer membrane. *J Biol Chem* **273**, 26310–26316.

PAPER IV

Weakening effect of cell permeabilizers on Gram-negative bacteria causing biodeterioration

In: Applied and Environmental Microbiology 2006.
Vol. 72, No. 7, pp. 4695–4703.
Reprinted with permission from the publisher.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2006, p. 4695–4703 Vol. 72, No. 7
0099-2240/06/$08.00+0 doi:10.1128/AEM.00142-06
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

Weakening Effect of Cell Permeabilizers on Gram-Negative Bacteria Causing Biodeterioration

H.-L. Alakomi,[1]* A. Paananen,[1] M.-L. Suihko,[1] I. M. Helander,[1,2] and M. Saarela[1]

Biotechnology, VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Espoo,[1] and Division of Microbiology, Department of Applied Chemistry and Microbiology, P.O. Box 56, FI-0014 University of Helsinki,[2] Finland

Received 19 January 2006/Accepted 14 March 2006

Gram-negative bacteria play an important role in the formation and stabilization of biofilm structures on stone surfaces. Therefore, the control of growth of gram-negative bacteria offers a way to diminish biodeterioration of stone materials. The effect of potential permeabilizers on the outer membrane (OM) properties of gram-negative bacteria was investigated and further characterized. In addition, efficacy of the agents in enhancing the activity of a biocide (benzalkonium chloride) was assessed. EDTA, polyethylenimine (PEI), and succimer (meso-2,3-dimercaptosuccinic) were shown to be efficient permeabilizers of the members of *Pseudomonas* and *Stenotrophomonas* genera, as indicated by an increase in the uptake of a hydrophobic probe (1-*N*-phenylnaphthylamine) and sensitization to hydrophobic antibiotics. Visualization of *Pseudomonas* cells treated with EDTA or PEI by atomic force microscopy revealed damage in the outer membrane structure. PEI especially increased the surface area and bulges of the cells. Topographic images of EDTA-treated cells were compatible with events assigned for the effect of EDTA on outer membranes, i.e., release of lipopolysaccharide and disintegration of OM structure. In addition, the effect of EDTA treatment was visualized in phase-contrast images as large areas with varying hydrophilicity on cell surfaces. In liquid culture tests, EDTA and PEI supplementation enhanced the activity of benzalkonium chloride toward the target strains. Use of permeabilizers in biocide formulations would enable the use of decreased concentrations of the active biocide ingredient, thereby providing environmentally friendlier products.

Stone monuments are subject to the deteriorative and degradative action of the environment and living organisms (12, 27, 45). Growing concern for the preservation of cultural heritage has boosted research on the biological attack on historical buildings (11, 15, 28). Biodeterioration processes result from complex interactions of surface-invading microbes with each other as well as with the surface material. Additionally, environmental factors and physiochemical properties of the surface material in question (e.g., porosity) determine the aggressiveness of the deterioration process and, as a consequence, its result (11). Besides being a potential cause of decay, soiling induced by biological growth results in aesthetical disfiguration of the stone and causes both physical and chemical damage on stone monuments.

Understanding the complex microbial ecosystem of building materials is a prerequisite for controlling the growth of microbial species causing biodegradation. Organisms present on stone monuments include photolithoautotrophs, such as algae and cyanobacteria, chemolithoautotrophic bacteria, mosses, and higher plants (11, 15, 45). The phototrophs algae and cyanobacteria have been considered the primary colonizers of building surfaces, conditioning the surfaces and excreting nutrients and growth factors for heterotrophic microbes (11). A majority of the microbes persist on building surfaces within complex microbial communities and a structured biofilm ecosystem (18), which provides shelter for the microbes. In addition, the endolithic environment, the pore space of rocks, has been reported to be a microhabitat giving protection from intense solar radiation and desiccation as well as providing mineral nutrients, rock moisture, and growth surface (42). Extracellular polysaccharides especially play various roles in the structure and function of different biofilm communities: e.g., excluding and/or influencing the penetration of antimicrobial agents and providing protection against a variety of environmental stresses, such as UV radiation, pH shifts, osmotic shock, and desiccation (10, 13, 35). Besides phototrophic cyanobacteria, many other gram-negative bacterial species, e.g., members of *Pseudomonas*, *Stenotrophomonas*, and *Sinorhizobium* genera, have been isolated from biodeteriorated stone samples (11, 38). Since members of these genera are potential extracellular polysaccharide produces (35), prevention of their growth or adhesion to stone materials would provide means to diminish biofilm formation on stone surfaces.

An additional factor making the prevention of gram-negative bacterial growth extremely difficult is related to the structure of the gram-negative cell envelope (14, 26, 37). The outer membrane (OM) of gram-negative bacteria acts as a permeability barrier that is able to exclude macromolecules and hydrophilic substances, thereby being responsible for the intrinsic resistance of these bacteria to antimicrobial compounds (14, 30, 31). In gram-negative bacteria, the barrier function of the OM is mainly due to the presence and features of lipopolysaccharide (LPS) molecules in the outer leaflet of the membrane, along with various multidrug efflux pumps that also contribute to the resistance of the cells (31, 32, 33). *Pseudomonas* species especially have been reported to be resistant to many biocides and antimicrobial agents (36, 37). According to Walsh et al. (43), the inner core phosphates of *P. aeruginosa* appear to play a key role in the intrinsic drug resistance of this bacterium.

* Corresponding author. Mailing address: VTT Technical Research Centre of Finland, Biotechnology, P.O. Box 1000, FI-02044 VTT, Espoo, Finland. Phone: 358 20 722 7158. Fax: 358 20 722 7071. E-mail: Hanna-Leena.Alakomi@vtt.fi.

TABLE 1. Target strains used in the study

Strain	VTT code	Identification[b]	Isolation site
Sinorhizobium morelense	E-022105	100% *S. morelense*^{T}	Marble on fresco, Italy
Pseudomonas sp.[a]	E-022106	98.3% *P. putida*^{T}/"*P. gingerii*"^{T}/*P. fuscovaginae*^{T}/*P. asplenii*^{T}	Marble on fresco, Italy
Stenotrophomonas nitritireducens	E-022107	99.8% *S. nitritireducens*	Marble on fresco, Italy
Pseudomonas sp.[a]	E-022217	98.9% *P. jessenii*^{T}, 98.0% *P. putida*^{T}	Marble on fresco, Italy
Pseudomonas sp.[a]	E-052906	98.8% *P. mendocina*	Sandstone, Scotland
Pseudomonas sp.[a]	E-052911	98.9% *P. mandelii*^{T}	Sandstone, Scotland

[a] Probably a new species.
[b] GenBank accession numbers for partial 16S rRNA gene sequences are, in descending order, DQ465005, DQ465006, DQ465007, DQ465008, DQ465009, and DQ465010.

Although the OM of gram-negative bacteria protects the cells from many external agents, it is possible to specifically weaken it by various agents, collectively called permeabilizers, which disintegrate the LPS layer and increase the permeability of the OM (40). The classical example is the chelator EDTA, which sequesters divalent cations that contribute to the stability of the OM by providing electrostatic interactions with proteins and LPS (2, 40). Besides EDTA, a number of other permeabilizers are known, some of which act quite differently. Polyethyleneimine (PEI), a cationic polymer, has been recognized as a permeabilizer acting by intercalating into the OM rather than releasing LPS (19, 20). Succimer (meso-2,3-dimercaptosuccinic; DMSA) is an active heavy-metal-chelating agent used, e.g., to treat lead poisoning in humans (16). Bansal-Mutalik and Gaikar (8) reported that sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) was capable of permeabilizing *Escherichia coli* cells and causing selective enzyme (penicillin acylase) release. Nitrilotriacetic acid (NTA) is a complexing agent of the same general type as EDTA, and it has been reported to be a permeabilizer and to increase the sensitivity of gram-negative bacteria to hydrophobic antibiotics (5, 40).

Mechanistic studies on the action of antimicrobial chemicals advance our understanding of their potential application. The objective of this study was to determine and characterize the effect of selected permeabilizers on the OM of environmental gram-negative bacteria isolated from biodeteriorated surfaces. We especially wanted to study the activity of PEI on the OM of *Pseudomonas*. To our knowledge, this is the first study using atomic force microscopy (AFM) to study the antimicrobial mechanisms of PEI and EDTA, well-known permeabilizers against *Pseudomonas.* Furthermore, the effect of permeabilizers on the activity of a biocide, benzalkonium chloride, was studied.

MATERIALS AND METHODS

Chemicals. HEPES, n-heptadecanoic acid methyl ester, 1-*N*-phenylnaphthylamine (NPN), polyethylenimine (PEI; mean molecular mass, 70 kDa), meso-2,3-dimercaptosuccinic acid (DMSA), nitrilotriacetic acid (NTA), sodium bis-(2-ethylhexyl) sulfosuccinate (AOT), and benzalkonium chloride (BC) were from Sigma-Aldrich (Steinheim, Germany); EDTA was from Riedel-de-Haen (Seelze, Germany). A stock solution of NPN (0.5 M) was prepared in acetone and diluted to 40 μM into 5 mM HEPES (pH 7.2) for the fluorometric assays. A stock solution of DMSA was prepared in ethanol and of NTA in 1 M NaOH. Solvents were included as controls in the experiments.

Bacterial strains. The bacterial strains (Table 1) used for the studies were

TABLE 2. NPN uptake induced by EDTA, PEI, DMSA, NTA, AOT, and BC

Strain	$MgCl_2$ (mM) concn	Relative fluorescence (±SD) with the following additive to assay buffer[a]: Control	EDTA (1 mM)	EDTA (0.1 mM)	PEI (10 μg ml^{-1})	DMSA (1 mM)	NTA (1 mM)	AOT (1 mM)	BC (0.001%)
Sinorhizobium morelense									
E2105	0	467 ± 72	443 ± 103	415 ± 192	422 ± 16	483 ± 74	327 ± 151	366 ± 70	512 ± 79
E2105	1	256 ± 45	428 ± 78	260 ± 63	257 ± 39	494 ± 67	253 ± 49	333 ± 5*	410 ± 15**
Stenotrophomonas nitritireducens									
E2107	0	208 ± 29	303 ± 50*	338 ± 60	293 ± 19*	359 ± 89	287 ± 64	264 ± 44	348 ± 36**
E2107	1	78 ± 12	157 ± 24	76 ± 13	189 ± 12**	324 ± 70*	65 ± 11	199 ± 54	280 ± 69**
Pseudomonas sp.									
E2106	0	185 ± 13	354 ± 16***	284 ± 36	324 ± 32**	367 ± 77	265 ± 72	143 ± 9	377 ± 38**
E2106	1	122 ± 11	174 ± 49	130 ± 52	194 ± 40	332 ± 71	150 ± 34	127 ± 12	310 ± 49**
E2217	0	54 ± 29	286 ± 26***	128 ± 20	278 ± 15**	265 ± 50*	65 ± 8	148 ± 2**	265 ± 55***
E2217	1	72 ± 5	56 ± 27	67 ± 6	77 ± 1	211 ± 50**	63 ± 6	122 ± 51*	119 ± 8*
E2906	0	223 ± 53	525 ± 106**	483 ± 76***	390 ± 47**	471 ± 91**	249 ± 58	327 ± 78**	455 ± 21**
E2906	1	87 ± 15	160 ± 47	86 ± 17	269 ± 51*	468 ± 83**	77 ± 14	190 ± 45**	360 ± 9**
E2911	0	143 ± 55	405 ± 73***	352 ± 92***	268 ± 17**	300 ± 42***	120 ± 33	237 ± 100	332 ± 23***
E2911	1	92 ± 13	137 ± 40	100 ± 10	102 ± 40	193 ± 42**	91 ± 11	112 ± 73	143 ± 12

[a] The pH of all treatments was 7.0 ± 0.2, except for the pH of the DMSA treatment, which was 4.5 ± 0.2. *, $P < 0.02$ compared with the control; **, $P < 0.01$ compared with the control; ***, $P < 0.001$ compared with the control.

TABLE 3. Effect of PEI on the susceptibility of the target strains to selected antibiotics as determined by the agar diffusion method

Strain and antibiotic	Diam of inhibition zone (mm) with PEI concn (μg ml^{-1}) of:			
	0	25	50	250
Sinorhizobium morelense E2105				
Clindamycin, 25 μg	21	20	20	ND[a]
Rifampin, 30 μg	36	40	42	ND
Novobiocin, 100 μg	31	38	38	ND
Erythromycin, 78 μg	25	25	22	ND
Fucidin, 100 μg	20	23	26	ND
Pseudomonas sp. strain E2106				
Clindamycin, 25 μg	0	0	0	0
Rifampin, 30 μg	22	24	29	36
Novobiocin, 100 μg	0	12	20	30
Erythromycin, 78 μg	0	0	15	20
Fucidin, 100 μg	0	12	17	18
Stenotrophomonas nitritireducens E2107				
Clindamycin, 25 μg	12	17	12	ND
Rifampin, 30 μg	35	41	40	ND
Novobiocin, 100 μg	0	0	0	ND
Erythromycin, 78 μg	21	22	22	ND
Fucidin, 100 μg	17	23	22	ND

[a] ND, no data (no growth on agar plates supplemented with 250 μg ml^{-1} of PEI).

isolated from biodeteriorated mineral materials and deposited at the VTT culture collection as *Sinorhizobium morelense* (=*Ensifer adhaerens*) VTT E-022105 (later E2105); *Pseudomonas* sp. strains VTT E-022106 (E2106), E-022217 (E2217), E-052906 (E2906), and E-052911 (E2911); and *Stenotrophomonas nitritireducens* E-022107 (E2107). They were identified by partial 16S rRNA gene sequencing according to Saarela et al. (38). The working cultures were stored at −70°C and cultivated on trypticase soy agar (TSA; Oxoid, Basingstoke, United Kingdom) at 25°C. For permeability assays, cells were grown in Luria-Bertani broth (LB) as described by Helander et al. (19). Cultivations were carried out at 25°C with shaking (150 rpm, unless otherwise stated). Growth was monitored by measuring the A_{630} with a Multiskan MCC/340 spectrophotometer (ThermoLabSystems, Helsinki, Finland). Further details of cell treatments are given below under various experimental settings.

Permeability assays. Two different methods were utilized to determine permeability properties of the OM: (i) NPN uptake and (ii) susceptibility to hydrophobic antibiotics.

(i) NPN uptake. NPN uptake by bacterial suspensions was measured using black fluorotiter plates (Catalog no. 9502 867; ThermoLabSystems, Helsinki, Finland) and the automated fluorometer Fluoroskan Ascent FL (ThermoLabSystems) as described earlier (1, 21). Briefly, cells grown to an A_{630} of 0.5 ± 0.02 were deposited by centrifugation at room temperature for 10 min at 1,000 × *g* and suspended into a half volume of 5 mM HEPES buffer (pH 7.2). Aliquots (100 μl) of this cell suspension were pipetted into fluoroplate wells, which contained NPN (10 μM) and, as test substances, either EDTA (1.0 and 0.1 mM), PEI (10 μg ml^{-1}), DMSA (1 mM), AOT (1 mM), or HEPES buffer (control) to make up a total volume of 200 μl. If desired, $MgCl_2$ was added to the cell suspension before addition of NPN. Fluorescence was monitored within 3 min from four parallel wells per sample (excitation, 355 nm; half bandwidth, 38 ± 3 nm; emission, 402 nm; half bandwidth, 50 ± 5 nm). Each assay was performed at least three times.

(ii) Antibiotic susceptibility and growth inhibition tests. The susceptibility of bacterial cultures to hydrophobic antibiotics was tested with the agar diffusion method on Iso-Sensitest agar (Oxoid, Basingstoke, Hampshire, England) with or without PEI supplementation (5 to 250 μg ml^{-1}) using Neo-Sensitab discs (erythromycin, novobiocin, clindamycin, fucidin, and rifampin; Rosco Diagnostica, Taastrup, Denmark). The diameters of inhibition zones were measured after incubation of the plates at 25°C for 24 and 48 h. All determinations were performed with two replicates, and results are presented as mean values. Further susceptibility tests with PEI and combinations of PEI and novobiocin were performed in liquid cultures as described by Helander et al. (19) with an automated turbidometer (Bioscreen C; ThermoLabSystems). Microbiological growth curve data was collected and analyzed with Research Express software (Transcalactic Ltd., Helsinki, Finland).

Results from the permeability assays were analyzed statistically using two-tailed unpaired Student's *t* tests to determine differences.

Atomic force microscopy (AFM) studies. *Pseudomonas* sp. strain E2106 cells grown in LB to an A_{630} of 0.8 ± 0.02 were deposited by centrifugation at room temperature for 10 min at 1,000 × *g* and washed with 10 mM Tris-HCl buffer (pH 7.2), and the optical density of the suspension was adjusted to an A_{630} of 0.5 ± 0.02 with the same buffer. Cells were harvested by centrifugation (1,000 × *g*, 10 min at room temperature). For the treatments resuspended into either buffer alone or buffer supplemented with 1 mM EDTA or 10 μg of PEI mg^{-1}, cells were treated at 25°C for 10 min with shaking (150 rpm), harvested by centrifugation (10,000 × *g*) in an Eppendorf microcentrifuge for 1 min at room temperature, washed with ultrapure water, harvested by centrifugation as described above, and resuspended into sterile ultrapure water. Analysis was done with duplicate cultures.

For AFM analysis, the treated cells were applied on a freshly cleaved mica surface and allowed to dry before imaging (9, 29). To determine the effect of the treatments on the cell membrane, an average of four images on different areas for each sample were imaged. The images were acquired in air under ambient conditions using a NanoScope IIIa Multimode AFM (Digital Instruments, Santa Barbara, CA) equipped with a "J"-scanner. The tapping mode was used with scan rates of 0.5 to 1.2 Hz and as little force as possible, and the ratio of set point amplitude and free amplitude was usually 0.8 to 0.9 with a target amplitude 1 V. Noncontact silicon cantilevers (NSC15/AlBS; μMasch) with the nominal resonance frequency of 350 kHz and a tip radius better than 10 nm were used. The topography and phase-contrast images were captured simultaneously. The phase-contrast image shows the phase difference between the oscillations of the cantilever-driving piezo and the detected oscillations. Nanoscope III 5.12r2 software (Digital Instruments) was used in image processing, which only included flatten-

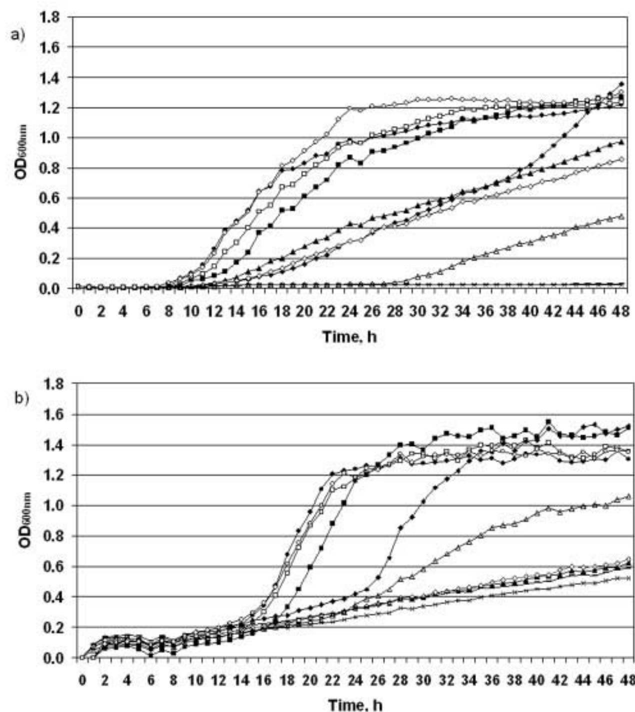


FIG. 1. Enhancement of susceptibility of *Pseudomonas* sp. strain E2106 (a) and *Stenotrophomonas nitritireducens* E2107 (b) to novobiocin by PEI. Bacterial growth, expressed as the optical density at 600 nm (OD_{600nm}), was measured for 48 h. Symbols: ○, control; ●, 10 μg of novobiocin ml^{-1}; □, 50 μg of novobiocin ml^{-1}; ■, 100 μg of novobiocin ml^{-1}; ◆, 10 μg of PEI ml^{-1}; ▲, 50 μg of PEI ml^{-1}; ◇, 100 μg of PEI ml^{-1}; Δ, 10 μg of novobiocin and 10 μg of PEI ml^{-1}; _, 10 μg of novobiocin and 50 μg of PEI ml^{-1}; ×, 10 μg of novobiocin and 100 μg of PEI ml^{-1}.

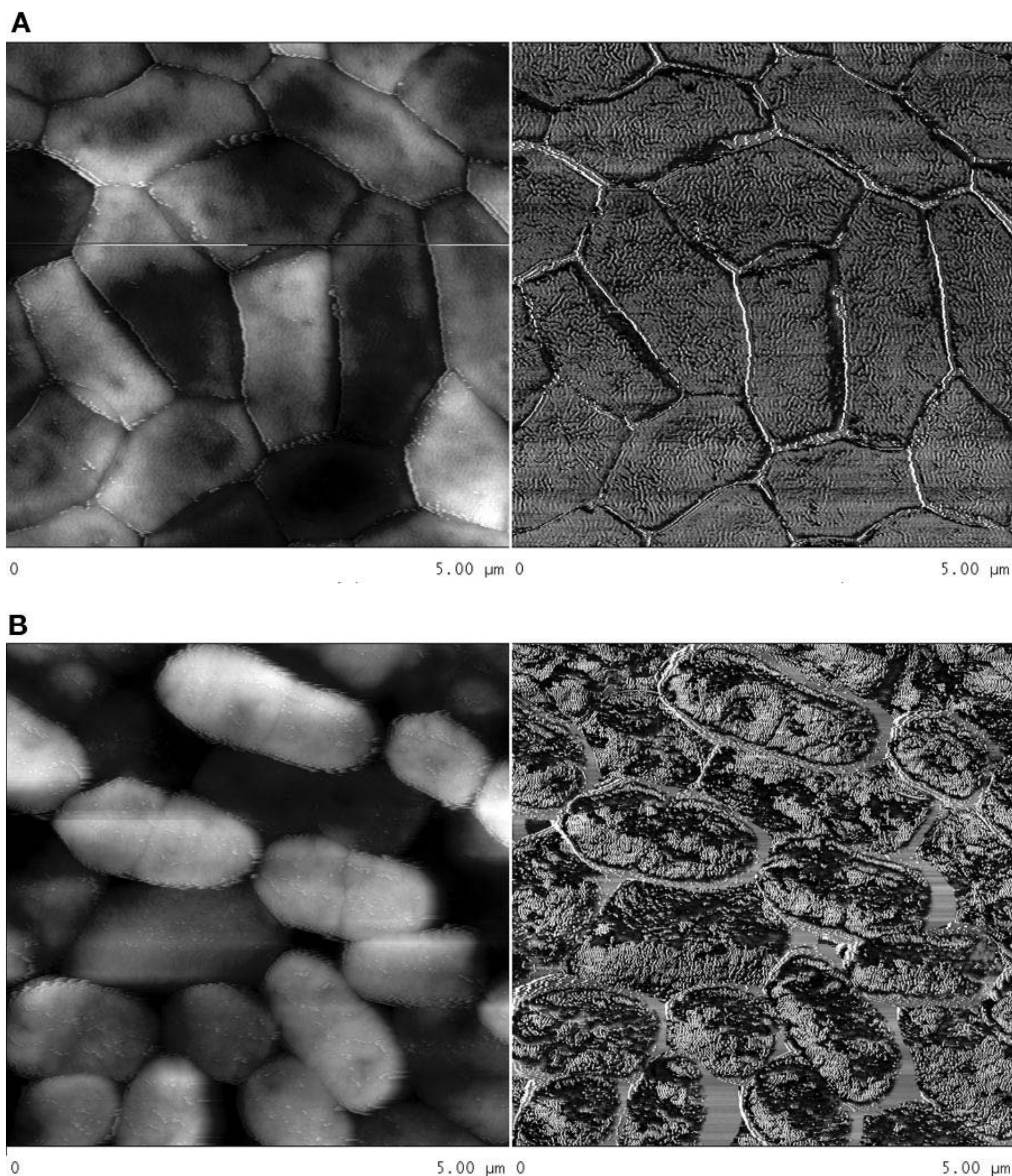


FIG. 2. Surface of control (A)-, 1 mM EDTA (B)-, and 10 μg ml^{-1} PEI (C)-treated *Pseudomonas* sp. strain E2106 cells visualized by atomic force microscopy (AFM). Left side, topographic image; right side, phase image.

ing in order to remove possible tilt in the image data. The average surface root-mean-square roughness of the treated cells was calculated with Nanoscope III 5.12r2 software from five replicate images with a resolution of 512 pixels.

Microtiter plate assay for biofilm formation (BF assay). Efficacy of EDTA, PEI, DMSA, and benzalkonium chloride on the biofilm formation of the target strains was assayed on 96-well microtiter plates (Nunclon 167008; Nalge Nunc International) with a protocol modified from Kolari et al. (23). Inoculum for the assay was grown overnight in trypticase soy broth (TSB; Oxoid; 25°C, 150 rpm) and diluted into TSB to obtain a cell density of 10^5 CFU ml^{-1}. Briefly, each of the microtiter plate wells was filled with a total volume of 250 μl with TSB, test agent (25 μl), and bacterial inoculum (25 μl). The plates were placed on a rotary shaker (120 rpm, 25°C) for 3 days. The wells were emptied, stained with 300 μl of crystal violet (4 g $liter^{-1}$ in 20% [vol/vol] methanol) for 3 min, washed three times under running tap water to remove planktonic cells, and allowed to dry in air. Stain retained by the biofilm was dissolved in ethanol (330 μl per well, 1 h), and the A_{595} was measured with a Multiskan MCC/340 spectrophotometer. All determinations were performed with three replicates, and results are presented as mean values. Each assay was performed three times.

Testing of the enhancement of in vitro antimicrobial activity. The capability of permeabilizers (EDTA and PEI) to enhance the activity of benzalkonium chloride was further assayed with an automated turbidometer (Bioscreen; Thermo-LabSystems) according to Raaska et al. (34). Briefly, each of the microtiter plate wells was filled with test agents (30 μl) and a dilution of the inoculum (30 μl; initial density, 10^4 CFU ml^{-1}), and then they were filled with TSB to a total

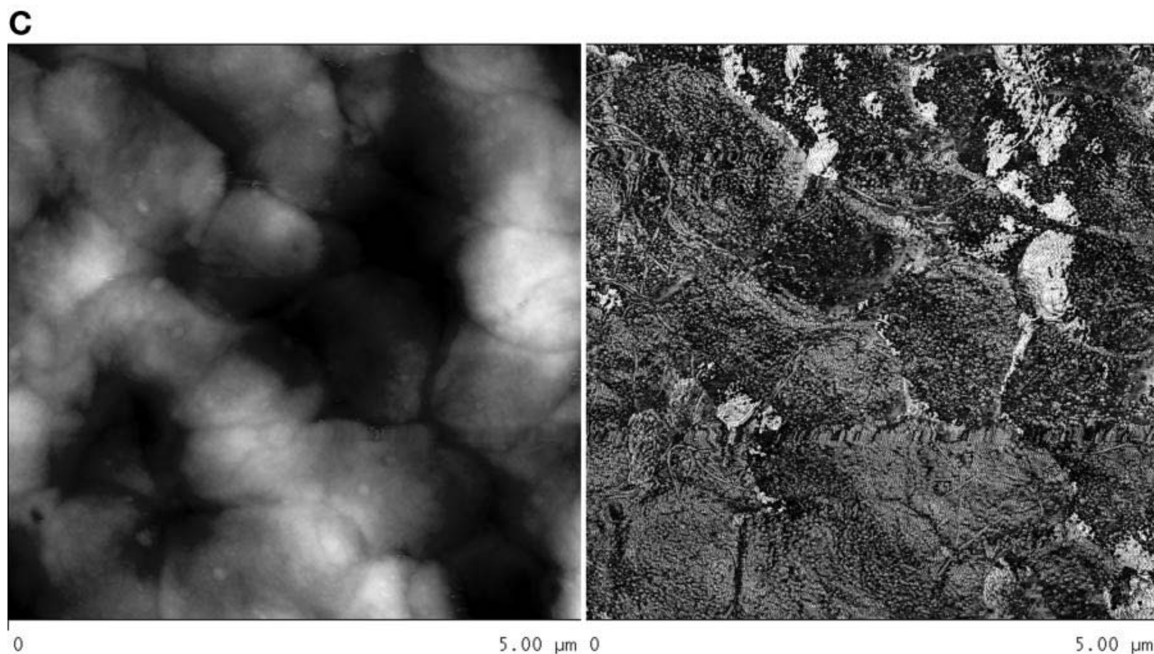


FIG. 2—*Continued.*

volume of 300 µl. The microtiter plates were incubated at 25°C for 48 h, and the optical density at 600 nm (OD_{600}) was measured every 10 min. Microbiological growth curve data were collected and analyzed with Research Express software (Transcalactic Ltd., Helsinki, Finland). The area under the growth curve was used as a measure of growth. All determinations were performed with five replicates, and results are presented as mean values. Each assay was performed three times.

Nucleotide sequence accession numbers. The sequences determined in the course of this work were deposited in GenBank under accession numbers DQ465005, DQ465006, DQ465007, DQ465008, DQ465009, and DQ465010.

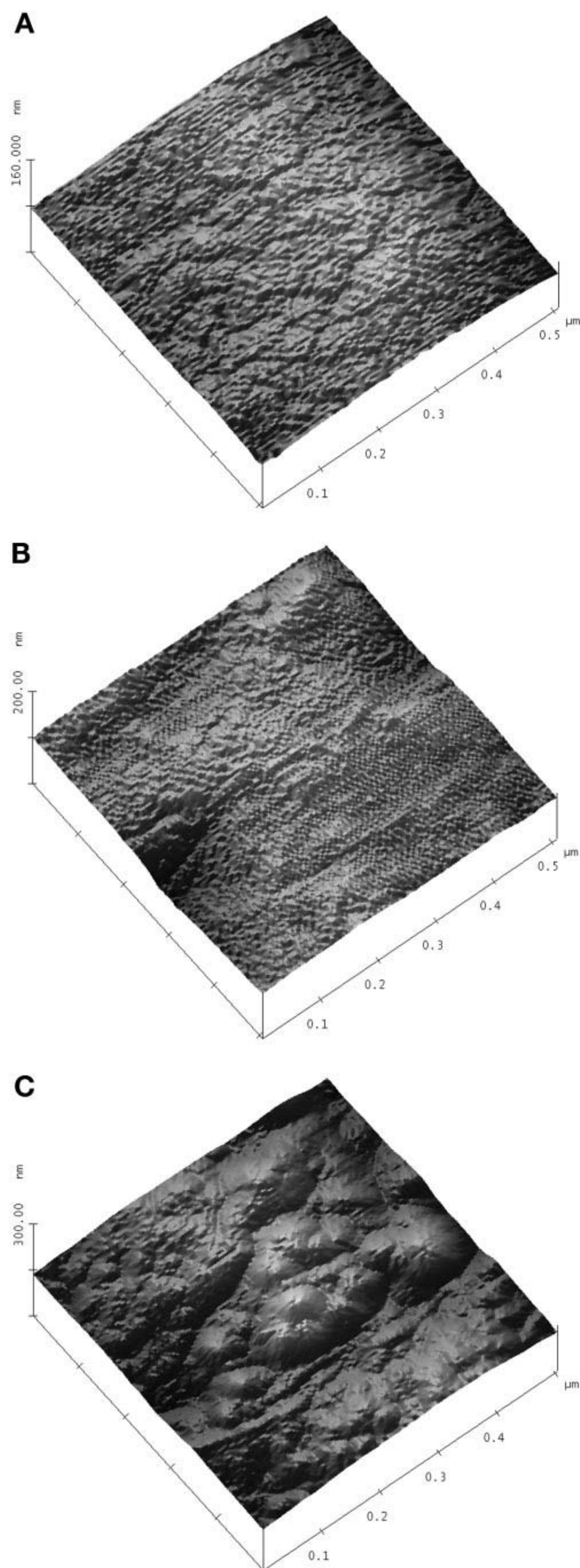
RESULTS

Effect of tested samples on the uptake of NPN. Environmental isolates were selected for NPN uptake studies in order to reveal possible changes in the OM permeability of gram-negative bacteria causing biodeterioration. NPN fluorescence is associated with the presence of this hydrophobic probe in a glycerophospholipid environment (20), and increased fluorescence values indicate weakening of the OM. The detailed results of the NPN uptake experiments with EDTA, PEI, DMSA, NTA, AOT, and benzalkonium chloride (BC) are presented in Table 2. EDTA (1 mM), PEI (10 $\mu g\ ml^{-1}$), and DMSA (1 mM) brought about a significantly higher NPN uptake than that of control treatments with all other strains except *S. morelense* E2105. Benzalkonium chloride (0.001%, wt/vol) weakened the outer membrane of the tested microbes, as indicated by a significant increase in the NPN uptake. Addition of 1 mM $MgCl_2$ into the buffer used in the NPN assay abolished the permeabilizing activities of 0.1 mM EDTA and diminished the permeabilizing activity of PEI. The OM-destabilizing activity of 1 mM EDTA and DMSA was only partially abolished by $MgCl_2$. DMSA (1 mM) supplementation resulted in pH 4.5 (±0.2) in the test assay, whereas for the other treatments the pH remained at 7.0 (±0.2). NTA at 1 mM concentrations did not significantly increase the NPN uptake of the target strains. AOT (1 mM) increased the NPN uptake of *Pseudomonas* sp. strain E2906, whereas NPN uptake of other microbes was only slightly affected.

Antibiotic susceptibility and growth inhibition tests. A sensitizing effect to hydrophobic antibiotics is one of the indications of OM-permeabilizing action. We tested the susceptibility of the target strains to a set of hydrophobic antibiotics (clindamycin, rifampin, novobiocin, erythromycin, and fucidin) by the agar diffusion method on plates containing different concentrations of PEI. The results are summarized in Table 3, which shows that PEI induced an increased susceptibility of *Pseudomonas* sp. strain E2106 to erythromycin, novobiocin, and fusidin. The susceptibility of *S. nitritireducens* E2107 was not, however, significantly enhanced by PEI addition on agar plate tests for these antibiotics (Table 3). PEI supplementation slightly increased the susceptibility of target strains to rifampin. Susceptibility of *S. morelense* E2105 was only slightly enhanced for novobiocin by PEI supplementation.

The effect of PEI on the growth of *Pseudomonas* sp. strain E2106 and *S. nitritireducens* E2107 was also tested using a Bioscreen automated turbidometer (Fig. 1). In the agar diffusion test, a larger concentration of PEI was required (>25 $\mu g\ ml^{-1}$) than in the suspensions (10 $\mu g\ ml^{-1}$) for the sensitization of *Pseudomonas* sp. strain E2106 cells to novobiocin (Fig. 1a). In suspension experiments, supplementation by 10 µg of PEI ml^{-1} enhanced the susceptibility of *Pseudomonas* sp. strain E2106 and *S. nitritireducens* E2107 cells to novobiocin (already with 10 $\mu g\ ml^{-1}$ of novobiocin). However, the growth of *S. nitritireducens* E2107 was not fully prevented by the combination of PEI and novobiocin even at higher concentrations tested (Fig. 1b).

AFM. Topographic images of the control *Pseudomonas* sp. strain E2106 cells revealed a compact and smooth surface without notable ruptures or pores on the cell surface (Fig. 2a).



Phase-contrast images of the control cells revealed that the hydrophilic surface was uniform. Magnification of the topographic images also revealed a uniform OM structure (Fig. 3a). The average surface root-mean-square roughness for the control cells was 2.06 ± 0.45. Figure 2b shows topographic and phase-contrast images of 1 mM EDTA-treated cells. The surfaces of cells visualized in topographic images were rough, and the outer membrane surface appeared damaged, indicating release of LPS and weakening of OM structure. Phase-contrast images revealed large areas with different hydrophilicity/hydrophobicity on the cell surface. The magnification of the topographic images showed extensive disruption of the LPS layer (Fig. 3b). The average surface roughness of EDTA-treated cells was 3.23 ± 0.49 ($P < 0.05$ compared to the control cells). The release of LPS from the surface of EDTA-treated cells resulted in large and irregularly shaped pits where the cytoplasmic membrane was revealed. The effect of PEI (Fig. 2c) was different from that of EDTA. Treatment of the cells with PEI flocculated the *Pseudomonas* cells, causing aggregation and adhesion of the cells. In addition, cells were swollen, with increased cell surface area and bulges. Magnification of the topographic image showed smooth OM surfaces with bulges and an increased surface roughness compared to that of control cells (Fig. 3c). Surface roughness of PEI-treated *Pseudomonas* cells was 7.48 ± 1.46 ($P < 0.001$ compared to the control cells).

Prevention of adhesion and biofilm formation on polystyrene plates. Figure 4 shows the quantification of biofilm formation by six environmental isolates in the BF assay. The most effective biofilm formers were *S. nitritireducens* E2107 and *Pseudomonas* sp. strains E2106, E2906, and E2911. EDTA at 1 mM concentration prevented biofilm formation of the tested strains. Benzalkonium chloride at a concentration of 0.01% significantly prevented biofilm formation of all tested strains compared to that of the control treatment. Also, a lower benzalkonium chloride concentration (0.001%) diminished the biofilm formation of the strains compared to that of control treatments, with *Pseudomonas* sp. strain E2106 being less affected than the other strains. DMSA at a concentration of 1 mM prevented the biofilm formation of *S. morelense* E2105 and *Pseudomonas* sp. strains E2906 and E2911. Supplementation by PEI (10 $\mu g\ ml^{-1}$) did not significantly decrease the biofilm formation compared to that of the control.

Enhancement of in vitro antimicrobial activity. The capability of selected permeabilizers to increase efficacy of benzalkonium chloride (BC) in suspensions is shown in Table 4. BC alone had a minor growth-inhibitory activity against the *Pseudomonas* sp. strains E2106 and E2217. Supplementation with PEI (10 $\mu g\ ml^{-1}$) significantly increased ($P < 0.001$) the activity of BC toward the tested *Pseudomonas* strains, whereas EDTA (0.1 mM) supplementation did not increase the activity. In the test assay, supplementation by PEI alone diminished the growth of *Pseudomonas* sp. strain E2106.

FIG. 3. Surface of control (A)-, 1 mM EDTA (B)-, and 10 $\mu g\ ml^{-1}$ PEI (C)-treated *Pseudomonas* sp. strain E2106 cells visualized by atomic force microscopy (AFM) in three-dimensional mode.

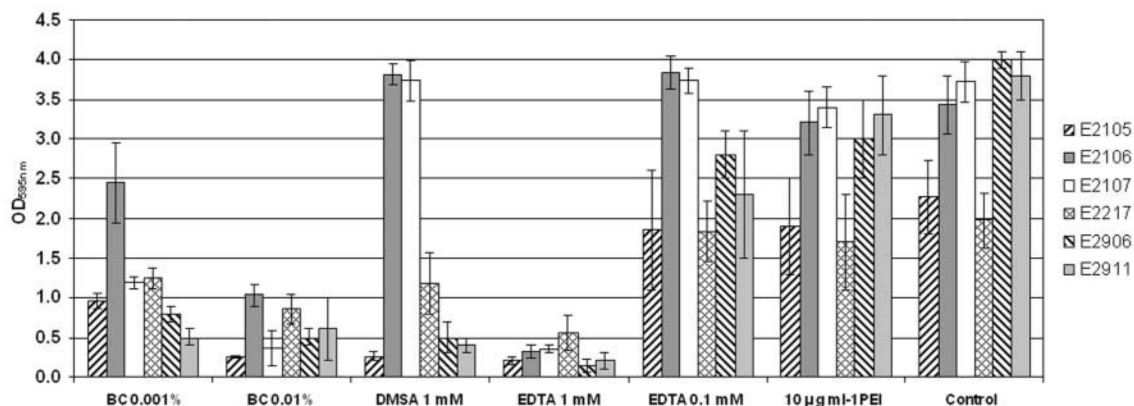


FIG. 4. Efficacy of different permeabilizers (BC, MDSA, EDTA, and PEI) on prevention of biofilm formation on polystyrene plates as determined by crystal violet staining.

DISCUSSION

Biocides must traverse the outer cell layer(s) of microbes to reach their target sites, usually present within microbial cells (36). Therefore, permeabilizers can enhance the activity of biocides and other antimicrobial agents, thus enabling application of a reduced amount of biocide. Among the potential permeabilizers examined in this study, EDTA, PEI, and succimer (DMSA) were shown to be efficient permeabilizers for the members of *Pseudomonas* and *Stenotrophomonas* genera, as indicated by the increase in the uptake of hydrophobic probe (NPN). This is in agreement with our earlier study where EDTA and DMSA destabilized the outer membrane of *Pseudomonas aeruginosa* E-97041, as indicated by an increased NPN uptake (3).

Intrinsic and acquired multidrug resistance in gram-negative bacteria is related to the synergy between limited OM permeability and energy-dependent multidrug efflux pumps (32). *S. morelense* is an opportunistic pathogen and has been reported to be highly resistant to several antibiotics (44). Our environmental *S. morelense* isolate, E2105, was sensitive to clindamycin, rifampin, novobiocin, erythromycin, and fucidin. Addition of PEI slightly increased the susceptibility of this strain to novobiocin. However, our target strain *S. morelense* E2105 seemed to have a weak OM structure, since in the NPN uptake assay the uptake values were already high in control cells and no statistically significant difference between various treatments was observed. This weak structure was likely related to the number of stabilizing divalent cations in the OM, since $MgCl_2$ addition stabilized the control cells but the permeabilizing activity of EDTA was not completely abolished by the $MgCl_2$ addition.

Pseudomonas species are able to degrade chloride compounds, and therefore they are not very sensitive to quaternary ammonium compounds (10, 17). Loughlin et al. (25) reported that *P. aeruginosa* cells generated stable resistance to benzalkonium chloride during passage in concentrations beneath the MIC of BC, and this resistance was also later retained in the absence of the disinfectant. In addition, a cross-resistance to the membrane-active antibiotic polymyxin B was also detected. In our studies, in the biofilm formation assay *Pseudomonas* sp. strain E2106 was the most resistant to benzalkonium chloride of the strains tested. EDTA and PEI enhanced the activity of benzalkonium chloride in suspension experiments toward *Pseudomonas*. In addition, our studies showed that benzalkonium chloride disintegrated the OM of the target cells, as indicated by an increased NPN uptake. Recently, it was reported that EDTA at high (50 mM) concentration caused rapid dispersion of *P. aeruginosa* cells from biofilms by chelation of several divalent cations that are required to stabilize the biofilm matrix (7).

Bansal-Mutalik and Gaikar (8) reported that AOT was capable of permeabilizing *Escherichia coli* cells and causing selective enzyme (penicillin acylase) release. In our study, AOT (1 mM) increased the NPN uptake of *Pseudomonas* sp. strain E2906, whereas NPN uptake of other microbes was only slightly affected. Nitrilotriacetic acid (NTA) has been reported to increase the sensitivity of gram-negative bacteria to hydrophobic antibiotics (5, 40). In our study, 1 mM NTA weakly destabilized *Pseudomonas* sp. strain E2106 and *S. nitritireducens* E2107 cells. However, NTA has been classified as possibly carcinogenic (4), and therefore it is not suitable to be used in biocide formulations intended for environmental applications, although it might have other application areas.

Succimer (DMSA) has been reported to be a potential remover of smear layers in dental applications (41). In our study, succimer was capable of destabilizing the OM of all tested

TABLE 4. Effect of permeabilizers on the enhancement of benzalkonium chloride (0.001%) activity in suspensions[a]

Tested sample	Area under the growth curve ± SD for:	
	Pseudomonas sp. strain E2106	*Pseudomonas* sp. strain E2217
Control	38 ± 1	41 ± 2
EDTA, 0.1 mM (pH 7)	35 ± 1*	43 ± 1
PEI, 10 μg ml^{-1} (pH 7)	20 ± 1***	40 ± 2
BC, 0.001% (wt/vol)	35 ± 0**	34 ± 2**
BC + EDTA	33 ± 3	32 ± 3**
BC + PEI	13 ± 5***	21 ± 2***

[a] Results are presented as areas under the growth curve (five replicates). *, $P < 0.02$ compared with the control; **, $P < 0.01$ compared with the control; ***, $P < 0.001$ compared with the control.

strains. In the NPN uptake assay, $MgCl_2$ addition only slightly abolished the OM disintegrating activity of DMSA, indicating that OM disintegrating activity of DMSA was only partially related to the removal of stabilizing divalent cations from the OM. Succimer is a hydroxy acid compound, and thereby part of the permeabilizing activity is related to the acidity and structure of the compound. Lactic acid, another hydroxy acid, has been shown to be a potent permeabilizer (1).

Polyethylenimine (PEI) is a weakly basic aliphatic polymer which is polycationic due to the presence of primary, secondary, and tertiary amino groups (6). PEIs are available in different molecular masses and forms, and they are widely utilized as protein and nucleic acid precipitants in process industry (39). Helander et al. (19) demonstrated that PEI is a potent permeabilizer of the OM of pathogenic gram-negative bacteria, as PEI sensitized *E. coli*, *P. aeruginosa*, and *Salmonella enterica* serovar Typhimurium to hydrophobic antibiotics and detergents. Helander et al. (20) also demonstrated that PEI intercalated in the OM and increased the membrane surface area without liberation of LPS-associated cell material from pathogenic gram-negative bacteria. Our study confirms that PEI is also capable of permeabilizing gram-negative environmental strains, representing *Pseudomonas* and *Stenotrophomonas* species, since significant NPN uptake and increased sensitivity to hydrophobic antibiotics was observed with these strains.

AFM images displayed massive changes on the OM of *Pseudomonas* sp. strain E2106 due to PEI treatment. This is not surprising, as *Pseudomonas* lipopolysaccharides typically are rich in phosphate groups (22, 43), and *Pseudomonas* cell surface is thus expected to bind polycationic PEI in large amounts. In PEI-treated cells, AFM images visualized the capability of PEI to intercalate in the OM and increase the membrane surface area. This observation is in agreement with the results of Helander et al. (20), who reported the same phenomenon in *Salmonella* by using transmission electron microscopy. Kotra et al. (24) studied the effect of EDTA on *E. coli* with AFM, and they reported that release of the LPS from the surface results in large and irregular-shaped pits where the peptidoglycan layer was exposed. Our AFM images from EDTA-treated *Pseudomonas* cells also revealed patchiness of the damaged OM structure. This nonuniform alteration of the OM by EDTA as revealed by AFM is in accordance with the classical findings that only a certain proportion of LPS can be released by EDTA, indicating the presence of structurally and electrostatically different subpopulations of LPS in the OM. The existence of such structurally distinct LPS populations in spatially separate areas of the OM, as discussed in more detail by Alakomi et al. (2), is further supported by our present findings with AFM.

Alternative and novel biocide formulations are needed to restrict the growth of harmful microbes in sites where traditional biocides or construction alternatives are ineffective or impossible to implement. The application of an effective biocide/permeabilizer combination could aid in the destruction of the microbial biofilms that cause the degradation while allowing the use of reduced concentrations of the biocide. In order to be able to enhance the activity of biocides, e.g., with permeabilizers, knowledge of the mechanism of permeabilizers and factors influencing their activity is essential. However, the efficacy of the formulated biocide products must be further evaluated on stone materials with complex microbial communities and in field trials under various environmental conditions. In addition, compatibility of the formulated products with commercial restoration products such as water repellents and consolidation agents has to be ensured.

ACKNOWLEDGMENTS

Päivi Lepistö, Tarja Nordenstedt, and Helena Hakuli are thanked for skillful technical assistance. We thank Historic Scotland for granting permission to sample the historic monuments.

This study has been carried out with financial support from the Commission of the European Communities, specific RTD program Energy, Environment, and Sustainable Development, contract EVK4-CT-2002-00098, project acronym BIODAM, "Inhibitors of biofilm damage on mineral materials."

This study does not necessarily reflect the Commission of the European Communities' views and in no way anticipates the Commission's future policy in this area.

REFERENCES

1. **Alakomi, H.-L., E. Skyttä, M. Saarela, T. Mattila-Sandholm, K. Latva-Kala, and I. M. Helander.** 2000. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Appl. Environ. Microbiol. **66:**2001–2005.
2. **Alakomi, H.-L., M. Saarela, and I. M. Helander.** 2003. Effect of EDTA on *Salmonella enterica* serovar Typhimurium involves a component not assignable to lipopolysaccharide release. Microbiology **149:**2015–2021.
3. **Alakomi, H.-L., N. Arrien, A. A. Gorbushina, W. E. Krumbein, I. Maxwell, C. McCullagh, P. Robertson, N. Ross, M. Saarela, J. Valero, M. Vendrell, and M. E. Young.** 2004. Inhibitors of biofilm damage on mineral materials (BIODAM), p. 399–406. *In* D. Kwiatkowski and R. Löfvendahl (ed.), Proceedings of the 10th International Congress on Deterioration and Conservation of Stone, vol. 1. International Council of Monuments and Sites, Stockholm, Sweden.
4. **Anonymous.** 1999. Some chemicals that cause tumors of the kidney or urinary bladder in the rodents and some other substances, p. 338. IARC monographs on the evaluation of carcinogenic risk of chemicals to humans, vol. 63. International Agency for Research of Cancer, Lyon, France.
5. **Ayres, H. M., D. N. Payne, J. R. Furr, and A. D. Russell.** 1998. Use of the Malthus-AT system to assess the efficacy of permeabilizing agents on the activity of antibacterial agents against *Pseudomonas aeruginosa*. Lett. Appl. Microbiol. **6:**422–426.
6. **Bahulekar, R., N. R. Ayyangar, and S. Ponrathnam.** 1991. Polyethyleneimine in immobilization of biocatalysts. Enzyme Microb. Technol. **13:**858–868.
7. **Banin, E., K. M. Brady, and E. P. Greenberg.** 2006. Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. Appl. Environ. Microbiol. **3:**2064–2069.
8. **Bansal-Mutalik, R., and V. G. Gaikar.** 2003. Cell permeabilization for extraction of penicillin acylase from *Escherichia coli* by reverse micellar solutions. Enzyme Microb. Technol. **32:**14–26.
9. **Bolshakova, A. V., O. I. Kiselyova, and I. V. Yaminsky.** 2004. Microbial surfaces investigated using atomic force microscopy. Biotechnol. Prog. **20:**1615–1622.
10. **Champanac, C., L. Pineau, A. Payard, G. Baziard-Mouysset, and C. Roques.** 2002. Interactions between biocide cationic agents and bacterial biofilms. Antimicrob. Agents Chemother. **5:**1469–1474.
11. **Crispim, C. A., and C. C. Gaylarde.** 2005. Cyanobacteria and biodeterioration of cultural heritage: a review. Microb. Ecol. **1:**1–9.
12. **Crispim, C. A., P. Gaylarde, and C. C. Gaylarde.** 2003. Algal and cyanobacterial biofilms on calcareous historic buildings. Curr. Microbiol. **46:**79–82.
13. **Davey, M. E., and G. A. O'Toole.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. **4:**847–867.
14. **Denyer, S. P., and J. Y. Maillard.** 2002. Cellular impermeability and uptake of biocides and antibiotics in gram-negative bacteria. J. Appl. Microbiol. **92**(Suppl):35S–45S.
15. **Dornieden, T., A. A. Gorbushina, and W. E. Krumbein.** 2000. Biodecay of cultural heritage as a space/time-related ecological situation—an evaluation of a series of studies. Int. Biodeterior. Biodegrad. **43:**261–270.
16. **Flora, S. J., M. Pande, and A. Mehta.** 2003. Beneficial effect of combined administration of some naturally occurring antioxidants (vitamins) and thiol chelators in the treatment of chronic lead intoxication. Chem.-Biol. Interact. **145:**267–280.
17. **Gilbert, P., and A. J. McBain.** 2003. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clin. Microbiol. Rev. **2:**189–208.
18. **Guerrero, R., M. Piqueras, and M. Berlanga.** 2002. Microbial mats and the search for minimal ecosystems. Int. Microbiol. **5:**177–188.

19. **Helander, I. M., H.-L. Alakomi, K. Latva-Kala, and P. Koski.** 1997. Polyethyleneimine is an effective permeabilizer of gram-negative bacteria. Microbiology **143:**3193–3199.
20. **Helander, I. M., K. Latva-Kala, and K. Lounatmaa.** 1998. Permeabilizing action of polyethyleneimine on *Salmonella typhimurium* involves disruption of the outer membrane and interactions with lipopolysaccharide. Microbiology **144:**385–390.
21. **Helander, I. M., and T. Mattila-Sandholm.** 2000. Fluorometric assessment of gram-negative bacterial permeabilization. J. Appl. Microbiol. **88:**213–219.
22. **Holst, O.** 1999. Chemical structure of the core region of lipopolysaccharides, p. 115–154. *In* H. Brade, S. M. Opal, S. N. Vogel, and D. C. Morrison (ed.), Endotoxin in health and disease. Marcel Dekker, Inc., New York, N.Y.
23. **Kolari, M., J. Nuutinen, F. A. Rainey, and M. Salkinoja-Salonen.** 2003. Colored moderately thermophilic bacteria in paper-machine biofilms. J. Ind. Microbiol. Biotechnol. **4:**225–238.
24. **Kotra, L. P., N. A. Amro, G.-Y. Liu, and S. Mobashery.** 2000. Visualizing bacteria at high resolution. ASM News **66:**675–681.
25. **Loughlin, M. F., M. V. Jones, and P. A. Lambert.** 2002. *Pseudomonas aeruginosa* cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. J. Antimicrob. Chemother. **49:**631–639.
26. **Maillard, J. Y.** 2002. Bacterial target sites for biocide action. J. Appl. Microbiol. **90**(Suppl)**:**16S–27S.
27. **Mansch, R., and E. Bock.** 1998. Biodeterioration of natural stone with special reference to nitrifying bacteria. Biodegradation **1:**47–64.
28. **May, E.** 2000. Comparative studies of microbial communities on stone monuments in temperate and semi-arid climates, p. 49–62. *In* O. Ciferri, P. Tiano, and G. Mastromei (ed.), Of microbes and art: the role of microbial communities in the degradation and protection of cultural heritage. Kluwer Academic/Plenum Publishers, New York, N.Y.
29. **Meincken, M., D. L. Holroy, and M. Rautenbach.** 2005. Atomic force microscopy study of the effect of antimicrobial peptides on the cell envelope of *Escherichia coli*. Antimicrob. Agents Chemother. **10:**4085–4092.
30. **Nikaido, H.** 2001. Preventing drug access to targets; cell surface permeability barriers and active efflux in bacteria. Cell Dev. Biol. **12:**215–223.
31. **Nikaido, H.** 2003. Molecular basics of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. **4:**593–656.
32. **Poole, K.** 2002. Outer membranes and efflux; the path to multidrug resistance in gram-negative bacteria. Curr. Pharm. Biotechnol. **2:**77–98.
33. **Poole, K.** 2004. Efflux-mediated multiresistance in gram-negative bacteria. Clin. Microbiol. Infect. **1:**12–26.
34. **Raaska, L., H.-L. Alakomi, M. Salkinoja-Salonen, and T. Mattila-Sandholm.** 1999. Antagonistic activity of *Staphylococcus* siderophores and chemical biocides against *Bacillus subtilis* in a paper-machine environment. J. Ind. Microbiol. Biotechnol. **22:**27–32.
35. **Robertson, E. B., and M. K. Firestone.** 1992. Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. Appl. Environ. Microbiol. **4:**1284–1291.
36. **Russell, A. D.** 2002. Antibiotic and biocide resistance in bacteria: introduction. J. Appl. Microbiol. **9**(Suppl)**:**1S–3S.
37. **Russell, A. D.** 2003. Similarities and differences in the response of microorganisms to biocides. J. Antimicrob. Chemother. **52:**750–763.
38. **Saarela, M., H.-L. Alakomi, M.-L. Suihko, L. Maunuksela, L. Raaska, and T. Mattila-Sandholm.** 2004. Heterotrophic microorganisms in air and biofilm samples from Roman catacombs with a special emphasis on actinobacteria and fungi. Int. Biodeterior. Biodegrad. **1:**27–37.
39. **Salt, D. E., S. Hay, O. R. T. Thomas, M. Hoare, and P. Dunhill.** 1995. Selective flocculation of cellular contaminants from soluble proteins using polyethylenimine; a study of several organisms and polymer molecular weights. Enzyme Microb. Technol. **17:**107–113.
40. **Vaara, M.** 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev. **56:**395–411.
41. **Vineeta, N., V. Singh, and S. Makkar.** 2001. Antimicrobial activity of dimercaptosuccinic acid (DMSA): a new chelating agent. J. Indian Soc. Pedod. Prev. Dent. **4:**160–163.
42. **Walker, J. J., J. R. Spear, and N. R. Norman.** 2005. Geobiology of a microbial endolithic community in the Yellowstone geothermal environment. Nature **434:**1011–1014.
43. **Walsh, A. G., M. J. Matewish, L. L. Burrows, M. A. Monteiro, M. B. Perry, and J. S. Lam.** 2000. Lipopolysaccharide core phosphates are required for viability and intrinsic drug resistance in *Pseudomonas aeruginosa*. Mol. Microbiol. **4:**712–727.
44. **Wang, E. T., Z. Y. Tan, A. Willems, M. Fernández-López, B. Reinhold-Hurek, and E. Martinez-Romero.** 2002. *Sinorhizobium morelense* sp. nov., a *Leucaena leucocephala*-associated bacterium that is highly resistant to multiple antibiotics. Int. J. Syst. Evol. Microbiol. **52:**1687–1693.
45. **Warscheid, T., and J. Braams.** 2000. Biodeterioration of stone: a review. Int. Biodeterior. Biodegrad. **4:**343–368.

Series title, number and report code of publication

VTT Publications 638
VTT–PUBS–638

Author(s)
Alakomi, Hanna-Leena

Title

Weakening of the Gram-negative bacterial outer membrane

A tool for increasing microbiological safety

Abstract

Gram-negative bacteria are harmful in various surroundings. In the food industy their metabolites are potential cause of spoilage and this group also includes many severe or potential pathogens, such as *Salmonella*. Due to their ability to produce biofilms Gram-negative bacteria also cause problems in many industrial processes as well as in clinical surroundings. Control of Gram-negative bacteria is hampered by the outer membrane (OM) in the outermost layer of the cells. This layer is an intrinsic barrier for many hydrophobic agents and macromolecules. Permeabilizers are compounds that weaken OM and can thus increase the activity of antimicrobials by facililating entry of hydrophobic compounds and macromolecules into the cell.

The work described in this thesis shows that lactic acid acts as a permeabilizer and destabilizes the OM of Gram-negative bacteria. In addition, organic acids present in berriers, i.e. malic, sorbic and benzoic acid, were shown to weaken the OM of Gram-negative bacteria. Organic acids can poteniate the antimicrobial activity of other compounds. Microbial colonic degradation products of plant-derived phenolic compounds (3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid, 3-phenylpropionic acid and 3-hydroxyphenylpropionic acid) efficiently destabilized OM of *Salmonella.* The studies increase our understanding of the mechanism of action of the classical chelator, ethylenediaminetetraacetic acid (EDTA). In addition, the results indicate that the biocidic activity of benzalkonium chloride against *Pseudomonas* can be increased by combined use with polyethylenimine (PEI). In addition to PEI, several other potential permeabilizers, such as succimer, were shown to destabilize the OM of Gram-negative bacteria. Furthermore, combination of the results obtained from various permeability assays (e.g. uptake of a hydrophobic probe, sensitization to hydtophobic antibiotics and detergents, release of lipopolysaccharide (LPS) and LPS-specific fatty acids) with atomic force microscopy (AFM) image results increases our knowledge of the action of permeabilizers.

ISBN
978-951-38-7014-0 (soft back ed.)
978-951-38-7015-7 (URL: http://www.vtt.fi/publications/index.jsp)

Series title and ISSN	Project number
VTT Publications 1235-0621 (soft back ed.) 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)	17342

Date	Language	Pages
April 2007	English, Finnish abstr.	95 p.+ app. 37 p.

Name of project	Commissioned by

Keywords	Publisher
Gram-negative bacteria, bacterial membranes, outer membrane, permeabilization, chelators, destabilizers, organic acids, EDTA, ethylenediaminetetraacetic acid, polyethylenimine	VTT P.O.Box 1000, FI-02044 VTT, Finland Phone internat. +358 20 722 4404 Fax +358 20 722 4374

Julkaisun sarja, numero ja raporttikoodi

VTT Publications 638
VTT–PUBS–638

Tekijä(t)
Alakomi, Hanna-Leena

Nimeke

Gram-negatiivisten bakteerien ulkokalvon heikentäminen
Keino parantaa mikrobiologista turvallisuutta

Tiivistelmä

Gram-negatiivisten bakteerien ryhmään kuuluu laaja joukko haittamikrobeja, esimerkiksi potentiaalisia patogeeneja (kuten salmonella) ja elintarvikkeissa esiintyviä pilaajamikrobeja. Monille Gram-negatiivisille bakteereille on ominaista kyky kasvaa pinnoilla ja muodostaa biofilmiä, minkä vuoksi ne aiheuttavat ongelmia prosessiteollisuudessa sekä kliinisissä ympäristöissä. Gram-negatiivisille bakteereille ominainen ulkokalvorakenne heikentää monien antimikrobisten yhdisteiden kulkeutumista solun sisään, minkä vuoksi Gram-negatiivisten bakteereiden kasvun estäminen on hankalaa. Permeabilisaattorit ovat yhdisteitä, jotka kykenevät vaurioittamaan Gram-negatiivisten bakteereiden ulkokalvorakennetta ja mahdollistavat hydrofobisten yhdisteiden, kuten antibioottien ja desinfektioaineiden, kulkeutumisen solun sisään, jossa ne pääsevät reagoimaan vaikutuskohteidensa kanssa.

Tässä työssä osoitettiin, että maitohappo ja lukuisat muut orgaaniset hapot (kuten bentsoe-, omena- ja sorbiinihappo, joita luontaisesti esiintyy marjoissa) heikensivät Gram-negatiivisten bakteerien ulkokalvoa. Orgaaniset hapot voivat tehostaa antimikrobisten yhdisteiden vaikutuksia. Suolistossa marjojen sisältämistä fenoliyhdisteistä muodostuvien mikrobien metaboliatuotteiden (fenyylipropioni- ja etikkahappojohdannaisia) osoitettiin heikentävän salmonellan ulkokalvoa ja herkistävän ne hydrofobisille antibiooteille. Lisäksi työssä selkiytettiin klassisen permeabilisaattorin, EDTA-kelaattorin, vaikutusmekanismeja salmonellaa kohtaan. Yhdistämällä useita permeabilisaattoriominaisuuksia mittaavia menetelmiä ja atomivoimamikroskopian antamat tulokset havaittiin, että polyetyleeni-imiini voimakkaasti vaurioitti *Pseudomonas*-bakteerien ulkokalvoa ja herkisti solut kvaternaariselle bentsalkoniumkloridi-biosidille. Yhdistämällä permeabilisaattoreiden käyttö antimikrobisiin yhdisteisiin voidaan vähentää ja laajentaa antimikrobisten yhdisteiden vaikutuksia Gram-negatiivisiin bakteereihin. Orgaanisten happojen salmonellan ulkokalvoa heikentävä vaikutus selittää osittain esimerkiksi maitohappobakteerien luontaisen kyvyn estää näiden haittamikrobien kasvua.

ISBN
978-951-38-7014-0 (nid.)
978-951-38-7015-7 (URL: http://www.vtt.fi/publications/index.jsp)

Avainnimeke ja ISSN	Projektinumero
VTT Publications 1235-0621 (nid.) 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)	17342

Julkaisuaika	Kieli	Sivuja
Huhtikuu 2007	Englanti, suomenk. tiiv.	95 s. + liitt. 37 s.

Projektin nimi	Toimeksiantaja(t)

Avainsanat	Julkaisija
Gram-negative bacteria, bacterial membranes, outer membrane, permeabilization, chelators, destabilizers, organic acids, EDTA, ethylenediaminetetraacetic acid, polyethylenimine	VTT PL 1000, 02044 VTT Puh. 020 722 4404 Faksi 020 722 4374

VTT PUBLICATIONS

618 Salo, Outi. Enabling Software Process Improvement in Agile Software Development Teams and Organisations. 2006. 149 p. + app. 96 p.

619 Hienonen, Risto, Keskinen, Jari & Koivuluoma, Timo. Reliability of materials for the thermal management of electronics. 113 p. + app. 31 p.

620 Talja, Heli. Asiantuntijaorganisaatio muutoksessa. 2006. 250 s. + liitt. 37 s.

621 Kutila, Matti. Methods for Machine Vision Based Driver Monitoring Applications. 2006. 82 p. + app. 79 p.

622 Pesonen, Pekka. Innovaatiojohtaminen ja sen vaikutuksia metsäteollisuudessa. 2006. 110 s. + liitt. 15 s.

623 Hienonen, Risto & Lahtinen, Reima. Korroosio ja ilmastolliset vaikutukset elektroniikassa. 2007. 243 s. + liitt. 172 s.

624 Leviäkangas, Pekka. Private finance of transport infrastructure projects. Value and risk analysis of a Finnish shadow toll road project. 2007. 238 p. + app. 22 p.

625 Kynkäänniemi, Tanja. Product Roadmapping in Collaboration. 2007. 112 p. + app. 7 p.

626 Hienonen, Risto & Lahtinen, Reima. Corrosion and climatic effects in electronics. 2007. 242 p. + app. 173 p.

627 Reiman, Teemu. Assessing Organizational Culture in Complex Sociotechnical Systems. Methodological Evidence from Studies in Nuclear Power Plant Maintenance Organizations. 2007. 136 p. + app. 155 p.

628 Kolari, Kari. Damage mechanics model for brittle failure of transversely isotropic solids. Finite element implementation. 2007. 195 p. + app. 7 p.

629 Communications Technologies. VTT's Research Programme 2002–2006. Final Report. Ed. by Markku Sipilä. 2007. 354 p.

630 Solehmainen, Kimmo. Fabrication of microphotonic waveguide components on silicon. 2007. 68 p. + app. 35 p.

631 Törrö, Maaretta. Global intellectual capital brokering. Facilitating the emergence of innovations through network mediation. 106 p. + app. 2 p.

632 Lanne, Marinka. Yhteistyö yritysturvallisuuden hallinnassa. Tutkimus sisäisen yhteistyön tarpeesta ja roolista suurten organisaatioiden turvallisuustoiminnassa. 2007. 118 s. + liitt. 81 s.

633 Oedewald, Pia & Reiman, Teemu. Special characteristics of safety critical organizations. Work psychological perspective. 2007. 114 p. + app. 9 p.

634 Tammi, Kari. Active control of radial rotor vibrations. Identification, feedback, feedforward, and repetitive control methods. Espoo 2007. 151 p. + app. 5 p.

635 Intelligent Products and Systems. Technology theme – Final report. Ventä, Olli (ed.). 2007. 304 p.

636 Evesti, Antti. Quality-oriented software architecture development. 2007. 79 p.

638 Alakomi, Hanna-Leena. Weakening of the Gram-negative bacterial outer membrane. A tool for increasing microbiological safety. 2007. 95 p. + app. 37 p.

Julkaisu on saatavana	Publikationen distribueras av	This publication is available from
VTT	VTT	VTT
PL 1000	PB 1000	P.O. Box 1000
02044 VTT	02044 VTT	FI-02044 VTT, Finland
Puh. 020 722 4404	Tel. 020 722 4404	Phone internat. + 358 20 722 4404
Faksi 020 722 4374	Fax 020 722 4374	Fax + 358 20 722 4374

ISBN 978-951-38-7014-0 (soft back ed.)
ISSN 1235-0621 (soft back ed.)

ISBN 978-951-38-7015-7 (URL: http://www.vtt.fi/publications/index.jsp)
ISSN 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)

VTT PUBLICATIONS 638 Weakening of the Gram-negative bacterial outer membrane Hanna-Leena Alakomi