

**Bile salt hydrolase in *Lactobacillus plantarum*:
functional analysis and delivery to the intestinal tract of the host.**

Jolanda Lambert

Promotor:

Prof. Dr. Willem M. de Vos
Hoogleraar Microbiologie
Wageningen Universiteit

Co-promotor:

Prof. Dr. Michiel Kleerebezem
Hoogleraar Bacterial Metagenomics
Wageningen Universiteit

Promotiecommissie:

Prof. Dr. Tjakko Abee
Wageningen Universiteit

Prof. Dr. Jerry Wells
Wageningen Universiteit

Prof. Dr. R. Paul Ross
Teagasc, Moorepark Food Research Centre, Ireland

Dr. Aat Ledeboer
Unilever, Vlaardingen

Dit onderzoek is uitgevoerd binnen de onderzoeksschool VLAG.

**Bile salt hydrolase in *Lactobacillus plantarum*:
functional analysis and delivery to the intestinal tract of the host.**

Jolanda Lambert

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. dr. M.J. Kropff
in het openbaar te verdedigen
op vrijdag 18 april 2008
des namiddags te vier uur in de Aula

J. M. Lambert. Bile salt hydrolase in *Lactobacillus plantarum*: functional analysis and delivery to the intestinal tract of the host.

PhD thesis. Wageningen University, The Netherlands, 2008.
With summary in Dutch.

ISBN: 978-90-8504-893-0

Abstract

In the liver of mammals, bile salts are synthesised from cholesterol and conjugated to either taurine or glycine. Following release into the intestine, conjugated bile salts can be deconjugated by members of the endogenous microbiota that produce an enzyme called bile salt hydrolase (Bsh). Bsh appears to play an important role in both host intestinal physiology and bacterial survival and persistence in the intestinal tract; especially lactobacilli have been suggested to be of importance for *in vivo* bile salt hydrolysis in the small intestine.

In this thesis, a functional analysis of Bsh in Gram-positive bacteria, and in particular, the model organism *Lactobacillus plantarum* WCFS1 was performed. In-depth investigation of the annotation of *bsh* genes in Gram-positive bacteria using a combination of *in silico* methods led to the re-annotation of eight conjugated bile acid hydrolase superfamily members in various lactobacilli. Furthermore, these analyses provided a robust methodology for accurate annotation of this enzyme superfamily. *L. plantarum* WCFS1 was previously predicted to contain four *bsh* genes (*bsh1*, *bsh2*, *bsh3*, and *bsh4*), but according to our *in silico* analyses, three of these genes appeared to be penicillin acylase-related.

To unravel the functionality of each of the separate *bsh* genes, the generation of multiple isogenic *bsh*-deletion strains was required. Therefore, a Cre-lox-based toolbox for the construction of multiple deletions and selectable-marker removal in Gram-positive organisms was designed and implemented in *L. plantarum* WCFS1. Using heterologous over-expression and multiple *bsh*-deletion derivatives of *L. plantarum* WCFS1, Bsh1 was shown to be the major bile salt hydrolase in this strain, where it appeared to be involved in glycodeoxycholic acid tolerance. Although these experiments validated the prediction that *bsh2*, *bsh3*, and *bsh4* do not encode true Bsh enzymes, the *in vivo* functionality of Bsh2, Bsh3, and Bsh4 was not entirely clarified. Bsh2, Bsh3, and Bsh4 appeared to encode enzymes with acylase activity possibly using penicillin-like chemicals as their preferred substrate.

To investigate the influence of Bsh-producing probiotics on host physiology, two modes of Bsh delivery to the small intestine were investigated in this work; delivery of Bsh activity by viable *L. plantarum* was compared to delivery using Bsh-whey protein/gum arabic microencapsulates in an *in vitro* model. The microencapsulates provided excellent protection of the enzyme during transit through gastric conditions, however, under pancreatin pressure during intestinal conditions, the Bsh enzyme was subject to proteolytic degradation. In contrast, *L. plantarum* was able to withstand both gastric and intestinal conditions, however, enzyme delivery levels are limited when compared to the capacity of microencapsulates. Finally, the influence of delivery of bile salt hydrolase activity by viable bacteria and whey protein/gum arabic microencapsulates on the host was investigated *in vivo* using a rat model.

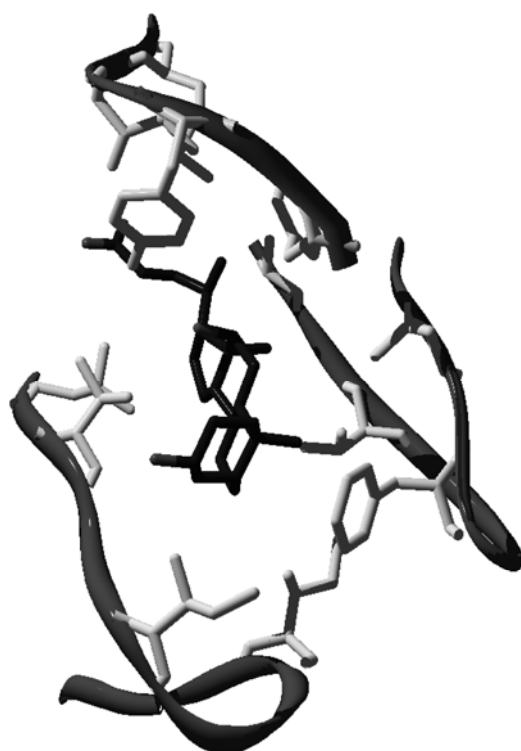
However, no effect of delivery of Bsh on the intestinal bile salt composition or mucin excretion was detected. These results may indicate that the physiological relevance and magnitude of bile salt hydrolase activity of probiotics in the small intestine is limited.

Index

1. Introduction and outline of this thesis	11
2. Improved annotation of conjugated bile acid hydrolase superfamily members in Gram-positive bacteria	33
3. A Cre- <i>lox</i> -based system for multiple gene deletions and selectable-marker removal in <i>Lactobacillus plantarum</i>	51
4. Functional analysis of conjugated bile acid hydrolase family members in <i>Lactobacillus plantarum</i> WCFS1	79
5. <i>In vitro</i> analysis of Bsh enzyme protection against enteric conditions by whey protein-gum arabic microencapsulation	107
6. <i>In vivo</i> modulation of host response by intestinal delivery of bile salt hydrolase activity	121
7. General discussion and concluding remarks	137
Nederlandse samenvatting	151
Nawoord.	153
<i>Curriculum vitae</i>	155
Publications and patents.	157
VLAG graduate school activities.	159

Chapter 1.

Introduction and outline of this thesis.



1. Introduction and outline of this thesis

INTRODUCTION

Intestinal microbiota. The microbiota of the intestine of humans and other mammals encompasses a myriad of microbes, and is dominated by low G+C Gram-positive bacteria. Between 10^3 to 10^8 bacteria per gram of intestinal content are present in the small intestinal tract, and this number rises to approximately 10^{11} bacteria per gram in the colon (Figure 1). Thereby, the total number of bacteria in the human intestine surpasses the total number of cells of the human body itself. To assess the activities and composition of the intestinal microbiota, culturing techniques have been employed extensively. However, many intestinal species appear to be unculturable to date. In addition, culturing techniques usually do not distinguish between injured, but physiologically active, and dead cells. It was shown that with flow cytometry using live/dead staining probes, viable, injured and dead cells can be distinguished, yielding relevant information on the physiological status of groups of intestinal bacteria (5, 14). Furthermore, a wide range of biochemical assays for common bacterial enzyme activities has been used for analysis of the activities of the microbiota (for review, see (109)). In addition, comparative genomics approaches can be used to predict the metabolic potential of the microbiota, e.g., using completely sequenced microbial genomes or libraries of large genomic DNA fragments from bacterial communities, often called metagenomic libraries (126). In addition, the *in vivo* expression of genes in bacteria has been studied. Examples include the use of microarrays from human *in vivo* samples of *L. plantarum* (34), or a resolvase-based IVET method that was used to identify promoters induced in *L. plantarum* during its passage through the gastro-intestinal tract of mice (21). In addition, various molecular typing methods have been used for analysis of the phylogeny of bacterial species present in the gastro-intestinal tract, such as protein electrophoresis and random amplification of polymorphic DNA (109). Many molecular typing methods for determination of the diversity of the microbiota are based on 16S rRNA sequences, such as sequencing and denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene amplicons, 16S rRNA gene fingerprinting, fluorescent *in situ* hybridisation (FISH), quantitative PCR, and 16S rRNA phylogenetic microarrays (e.g., the Human Intestinal Tract Chip (HitChip); (19, 35, 92, 109, 125, 126).

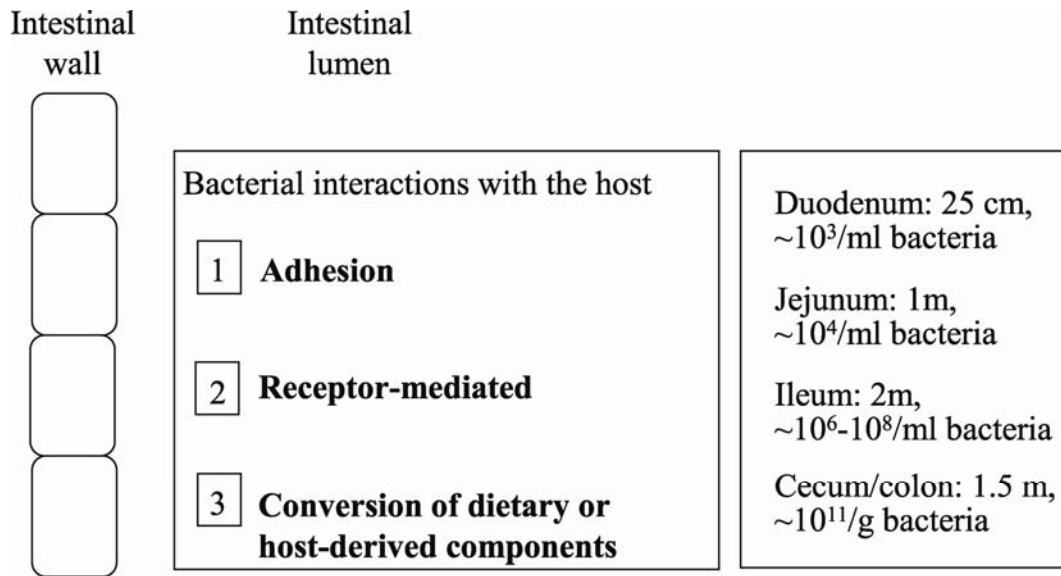


Figure 1. Interactions of intestinal bacteria with the human intestinal tract. The length of the various parts of the human intestine and its estimated bacterial content is indicated.

Host-microbe interactions. The intestinal microbiota has a profound influence on the host, which is exemplified by the marked differences found between germ-free animals and their conventional counterparts. For example, organ weights, cardiac output, intestinal wall thickness and motility, epithelial cell turnover and several immunological parameters are reduced or atrophic in germ-free animals when compared to the conventional situation (39, 50, 109).

Several modes of interaction between the host and intestinal microbes can be distinguished (Figure 1), such as direct attachment to mucosa or the mucin layer of the intestinal tract, molecular recognition of bacterial compounds by host receptors, and microbial conversion of host or diet-derived luminal factors. However, in many cases, the interactions occurring *in situ* will depend on more than a single molecular mechanism, and may also include additional mechanisms not encompassed by the modes of interaction presented here. Furthermore, our knowledge of the molecular basis of the interactions between the host and the microbiota is limited, although several studies have aimed to address this issue, such as experiments performed in germ-free animals (for review, see (39)), and among others, studies of the interaction between the host and specific bacteria, such as *L. plantarum* and *Akkermansia muciniphila* (20, 36, 78, 79). Therefore, the model of the modes of interaction between host and intestinal microbes presented here can only be seen as exemplary.

Attachment of bacteria to intestinal epithelial cells by direct adhesion most likely plays a key role in the capacity of microbial cells to colonize the intestine. The capacity to adhere to intestinal epithelial cells has been described for both intestinal pathogens as well as intestinal commensals. For example, several pathogens, such as *Escherichia coli* spp. (72, 124), *Salmonella* (6), and *Vibrio* (16) were shown to attach to epithelial cells through mannose-specific

adhesion. In addition, a mannose-specific adhesin was found in the non-pathogenic bacterium *Lactobacillus plantarum*, which is one of the *Lactobacillus* species frequently encountered in the human intestine (3, 85). Protection against intestinal infections by pathogenic bacteria through the activity of the resident microbiota may also depend on interactions between bacteria. For example, the microbiota protects the host from invasion by pathogens by filling all available ecological niches in the intestine, preventing growth of exogenous (pathogenic) organisms, which is a process often termed competitive exclusion, or by the secretion of specific antimicrobial substances, such as bacteriocins. Notably, bacteriocin production by *Lactobacillus salivarius* was found to be important in the protection of mice against *Listeria monocytogenes* infection (29). These experiments are examples of research on the molecular basis of host-microbe interactions. This type of research is indispensable for improving our understanding of the population dynamics and interactions taking place in the gastro-intestinal tract.

In a second mode of interaction, the host is influenced by binding of microbiota-derived molecules to host receptors, leading to receptor-mediated molecular host responses (Figure 1) (for review, see (120)). For example, immature dendritic cells, anchored between intestinal epithelial cells, recognize microbes by conserved pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), that recognize specific components of bacteria, such as lipopolysaccharide, lipoprotein or bacterial DNA (114, 116), and C-type lectins, such as DC-SIGN, that recognize carbohydrate structures on the cell surface of bacteria (122). The microbiota is thought to have a large impact on the immune system, which is clearly illustrated by the observation that the aspects of the immune system that are underdeveloped in germ-free animals are at least partially restored upon introduction of a microbiota (25, 59, 118). However, disbalance in the microbiota or disruption of the normal immunological response to the microbiota may lead to the development of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, where an abnormal inflammatory reaction against the natural microbiota is observed (98).

Possibly, the most prominent influence of the microbiota on host physiology is exerted through the capacity of intestinal microbes to convert dietary or host-derived components (Figure 1). For example, the microbiota contributes to the metabolism of the host in several ways, including the fermentation of non-digestible carbohydrates and mucus, the production of vitamin such as vitamin K, B₁₂, biotin, folic acid, and pantothenate, and the synthesis of amino acids from ammonia or urea (59). Accumulation of mucus in germ-free animals leads to a significant enlargement of the cecum (52), that can be reversed by the introduction of glycoside-hydrolysing bacteria, such as *Peptostreptococcus* (24). Furthermore, the microbiota does not only complement the metabolism of the host by fermentation of non-digestible polysaccharides (e.g., from plant material) to short chain fatty acids (SCFAs); colonization also

appears to modulate host genes that affect energy deposition in adipocytes (7). The contribution of the microbiota to host metabolism is clearly illustrated by the observation that germ-free mice are resistant to diet-induced obesity (8). Not all microbial activities contribute to host metabolism in a positive manner. For example, the bacterial putrefaction of proteins leads to the formation of potentially toxic and carcinogenic substances, such as ammonia, amines, phenols, thiols, and indols (50, 101). Another example of potentially harmful bacterial activities in the intestinal tract is the biotransformation of bile salts. To understand the influence of microbial bile salt biotransformations on host physiology, it is important to improve our knowledge of the differential responses of the intestinal mucosa to the natively produced bile salts compared to their derivatives that are generated through the activities of the intestinal microbiota.

Bile and bile salts. Since long, it has been recognized that the bile salts present in bile play an important role in the absorption and digestion of fats and lipid-soluble vitamins in vertebrates. Bile acids improve the solubility of dietary lipophilic substances in the watery environment of the small intestine, and enable digestion of triacylglycerols by lipases, liberating free fatty acids (1). Bile is produced in the liver by hepatocytes, followed by secretion into bile canaliculi, draining into the hepatic ducts. In many mammals, a proportion of the bile is stored into the gallbladder, where it is concentrated. Following food intake, the gallbladder is stimulated by the hormone cholecystokinin to secrete the bile into the duodenum (94). However, the gallbladder appears to be non-essential for proper functioning of bile, since some mammals, such as rat (but not all rodents), horse, most deer and most birds, do not possess a gallbladder. In these animals, a constant flow of relatively dilute bile is secreted directly into the small intestine (65).

Typically, mammalian bile contains bile salts (12 %) and phospholipids (4 %) (1), and a small amount of cholesterol and substances that cannot be eliminated via the urine, such as the protein-bound bilirubin, or drugs such as certain antibiotics and morphine. Bile salts and phospholipids form mixed micelles above their critical micellar concentration, which solubilise cholesterol. In addition, small amounts of the immunoglobulin IgA, mucus and tocopherol are present in bile, preventing bacterial growth and oxidative damage to the epithelium (56).

Bile salts are synthesized from cholesterol, and therefore can be seen as not only having a function in the digestion of fats, but also in the elimination of cholesterol. Most vertebrates synthesize C_{24} bile salts. However, in reptiles and some ancient mammals, such as elephant and sea cow, bile alcohols with more than 24 carbon atoms are formed (117). Biosynthesis of C_{24} bile salts occurs via the classical and the alternate biosynthetic pathway (1, 97). The classical pathway is operated entirely in the liver, whereas in the alternate pathway, the first steps of cholesterol conversion are carried out in other tissues, using

Introduction

oxysterols as intermediate molecules; the last steps of bile salt synthesis, however, are always carried out in the liver. The alternate pathway for synthesis of bile salts may play an important role in the elimination of excess cholesterol from tissues such as the brain; an impairment in brain cholesterol homeostasis is thought to be involved in several neurodegenerative diseases (17). The classical pathway, which is quantitatively the most important, involves the epimerisation of the 3 β -hydroxyl group of cholesterol, the elimination of the side chain, and saturation and hydroxylation of the steroid nucleus (1) (Figure 2).

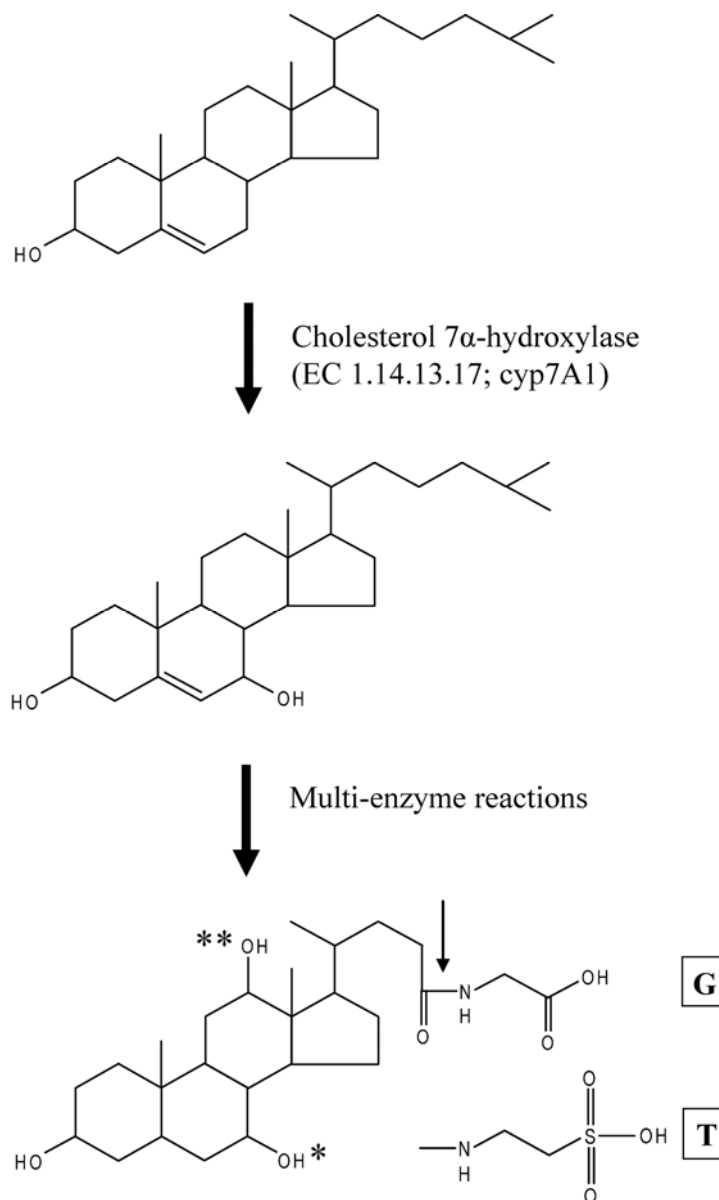


Figure 2. Biosynthesis of bile salts from cholesterol via the classical pathway. As an example, the structure of glycocholic acid (GC) is shown. The bond that is hydrolysed by Bsh-activity is indicated by an arrow. The OH-group that is absent in glycodeoxycholic acid (GDC) when compared to glycocholic acid is indicated with *. Likewise, the OH-group that is absent in glycochenodeoxycholic acid (GCDC) is marked with **; in lithocholic acid (LC), the two OH-groups marked with * and **, respectively, are absent. G, glycine; T, taurine.

Commonly, one or two hydroxyl groups are added to the bile salts, with the site of hydroxylation varying between species. Hydroxylation occurs on one side of the steroid nucleus, resulting in an amphiphatic molecule with detergent properties. In human, hydroxylation results in the formation of the bile salts cholic acid (C) and chenodeoxycholic acid (CDC) (Figure 2). Finally, the amphiphatic nature of the bile salts is further enhanced by the addition of glycine or taurine (Figure 2). The type of amino acid that is conjugated to the bile salts depends on animal species and can be influenced by the diet. Since taurine is abundantly available in meat, but not in plants, carnivores usually produce mainly taurine-conjugated bile salts, whereas herbivores usually produce mainly glycine-conjugated bile salts. Omnivores synthesize both glycine- and taurine-conjugated bile salts (1). In rats, the source of carbohydrates in the diet has been shown to have a major influence on the ratio of glycine and taurine conjugation of bile salts. However, evidence on the precise mechanism is conflicting and needs further investigation (43, 61-64).

Enterohepatic cycling of bile salts. Following excretion into the small intestine, conjugated bile salts are efficiently (~ 95 %) re-absorbed by epithelial cells of the terminal ileum (Figure 3). Putative transporters involved in the transport of bile salts have been described; for review, see (4, 73, 84, 115).

At physiological pH, conjugated bile salts, and especially taurine-conjugated bile salts, are membrane-impermeable anions. Therefore, conjugated bile salts are mainly recovered from the terminal ileum by active transport through the apical sodium dependent bile acid transporter (ASBT) (44, 53) and sodium independent organic anion transporting peptides (OATPs) (121). The transcellular transport of bile acids is mediated by the cytosolic ileal bile acid binding protein (IBABP) (76). Following transport from the epithelial cells into the portal blood via organic solute transporters (OSTs) (30), the bile salts are actively taken up into hepatocytes by the sodium taurocholate co-transporting peptide (NTCP) and OATPs (83). Subsequently, the bile salts are transported through the hepatic cells by binding proteins and resecreted by an ATP-dependent bile salt excretory pump (BSEP) (106) and multi drug resistance proteins (MRPs) (115). This process of secretion and re-absorption is called enterohepatic cycling (Figure 3). The rate of enterohepatic cycling greatly exceeds the rate of synthesis from cholesterol, providing an efficient way of both excreting lipophilic molecules from the liver and the digestion and uptake of fats and lipid-soluble vitamins in the small intestinal tract.

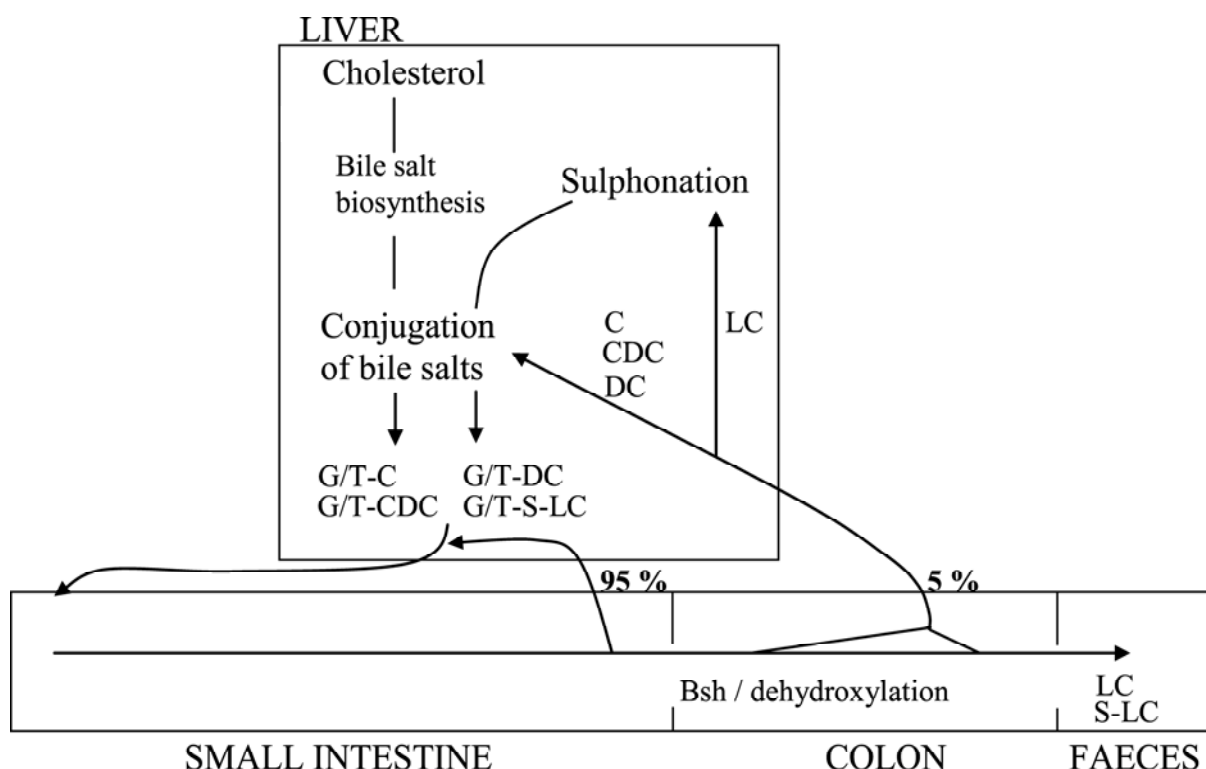


Figure 3. Schematic overview of the enterohepatic cycle of bile salts in human. The primary bile salts cholic acid (C) and chenodeoxycholic acid (CDC) are synthesized from cholesterol and conjugated to glycine (G) or taurine (T) in the liver. Subsequently, the bile salts are secreted into the small intestine. At the distal part of the small intestine, the majority of glycine- or taurine-conjugated C and CDC is actively reabsorbed into the portal blood and resecreted via the liver. A part of the primary, conjugated, bile salts C and CDC is deconjugated by the microbiota in the intestinal tract into free C and CDC by bile salt hydrolase (Bsh) activity, and subsequently, may be dehydroxylated into deoxycholic acid (DC) and lithocholic acid (LC), respectively. These bile salts are passively absorbed along the intestinal tract, reconstituted with glycine or taurine in the liver, and re-secreted into the small intestine. LC, however, is poorly soluble, and is partly excreted in faeces. The fraction of LC that is reabsorbed is sulphonated in the liver to a poorly reabsorbed form, re-secreted into the small intestine, and finally excreted in faeces.

Bile salt biotransformations. Some of the bile salts synthesized by the host, referred to as primary bile salts, are metabolized by the microbiota encountered in the intestinal tract. The first and obligatory step (10) in the majority of bile salt transformations is the deconjugation of the amino acid moiety of the bile salts by a bacterial enzyme called bile salt hydrolase (Bsh, or conjugated bile acid hydrolase [CBAH]). Subsequently, the quantitatively most important bile salt biotransformation is 7 α -dehydroxylation of the steroid nucleus, leading to the formation of bile salts referred to as secondary bile salts. This type of bile salt biotransformation has been attributed mainly to colonic activity of clostridia (95). In human, the dehydroxylation of the primary bile salts C and CDC results in the secondary bile salts deoxycholic acid (DC) and lithocholic acid (LC) (Figure 2). Another type of bile salt biotransformation that occurs to a much lower extent is oxidation and epimerization of the bile salt

hydroxyl groups, carried out by hydroxysteroid dehydrogenases that are found for example in *Clostridium*, *Bacteroides*, *Egerthella lenta*, *Escherichia coli*, *Peptostreptococcus productus*, and *Eubacterium aerofaciens* (95).

In contrast to conjugated bile salts, deconjugated bile salts are not charged at physiological pH and can be passively absorbed along the entire intestine (Figure 2). The rate of cell entrance is dependent on bile salt hydrophobicity. Thus, deconjugated primary and secondary bile salts enter the enterohepatic circulation as well, with the exception of LC, which is usually sulphated in the liver to a poorly re-absorbed bile salt and excreted in faeces. In some animals, such as rodents and prairie dogs (88), but not humans (15), it was found that the liver is able to convert secondary bile acids back into primary bile salts.

Impact of the bile salt-converting activity of the microbiota on the host. The generation of secondary bile salts by intestinal bacteria, which occurs after the deconjugation of primary bile salts, was shown to have a large impact on host physiology. Especially, elevated levels of the secondary bile salts DC and LC have been correlated with the development of colorectal cancer (for review, see (82)). Furthermore, the more hydrophobic (i.e., deconjugated and secondary) bile salts have been found to increase mucin excretion from gallbladder *ex vivo* tissues and cell lines and intestinal epithelial cell lines (55, 69-71, 99), which is thought to promote the nucleation of cholesterol gallstones. Since mucin is the major component of the protective mucous layer lining the intestine, an increase in mucin excretion can be seen as a response to the cytotoxicity of hydrophobic bile salts. Furthermore, increased mucin excretion could influence the nutritional environment encountered by the intestinal microbiota, and decrease intestinal transit time (100), eventually even resulting in diarrhoea. However, prolonged exposure of colonic cell lines to relatively low concentrations of hydrophobic bile salts was found to reduce mucin excretion (99). Mucin is the main constituent of the mucous layer lining the intestine. The mucous layer appears to prevent direct adhesion of gut bacteria to the epithelial surface (80), therefore, a reduction in mucin excretion could impair the barrier function of the mucous layer against irritants and pathogens.

Notably, the intake of several probiotic strains (i.e., bacterial strains conferring a specific health benefit to the host) has been linked to lowering of serum cholesterol levels (32, 87, 90, 91, 112). Several drugs are available to treat hypercholesterolemia, such as bile acid sequestrants, which are synthetic polymeric resins binding bile salts, draining the bile salt pool and increasing the demand for bile acid synthesis from cholesterol, and statins, which inhibit the enzyme HMG-CoA reductase that is involved in cholesterol synthesis. However, these agents often have side effects such as gastro-intestinal complaints and muscle cramps. Thus, the ingestion of probiotics may provide an attractive alternative. A partial explanation for the cholesterol-lowering effect of some bacterial strains may lie in their bile salt-hydrolyzing activity. Deconjugated bile salts are less soluble and therefore, are more prone to be extracted from the

enterohepatic cycle by precipitation and excretion in the faeces than the more soluble conjugated bile salts. Thereby, the demand for *de novo* bile salt synthesis from cholesterol would increase, leading to cholesterol lowering. In addition, deconjugated bile salts are not as efficient in solubilising lipids such as cholesterol, possibly lowering blood cholesterol levels by an impaired uptake of cholesterol from the diet. However, this also implicates that bile salt hydrolase activity might compromise normal lipid digestion. Indeed, in chicken, an increase in growth by antimicrobial supplementation was found to be related to a decrease in total microbial Bsh activity (and in particular, *Lactobacillus*) (40, 41, 51). However, this relation was not found in mice (9), indicating that intestinal Bsh activity does not always correlate to growth defects in the host.

Bacterial Bsh activity. Since bile salt deconjugation by intestinal bacteria is thought to have a large impact on host physiology (see above), it deserves special attention. The enzyme responsible for bile salt deconjugation, bile salt hydrolase (EC3.5.1.24), is expressed by a wide variety of Gram-positive bacteria, such as *Enterococcus faecium* (123), *Bifidobacterium* species (67, 108), *Clostridium* (28), *Lactobacillus* species (27, 38, 74, 75) and *Listeria monocytogenes* (37). Experimentally verified Pva-family proteins can be found for *Bacillus* species (89, 93) and *Listeria monocytogenes* (13) (for review, see (12)). Among Gram-negative bacteria, only *Bacteroides* was found to express bile salt hydrolase activity (66, 105). Bsh amino acid sequences resemble those of penicillin V acylases (Pva, EC3.5.1.11) and belong to the enzyme superfamily of linear amide C-N hydrolases (Pfam CBAH, PF02275). Some bacteria have been predicted to possess more than one Bsh-homologue, such as *L. plantarum* (68), *L. johnsonii* (38), *L. acidophilus* (81) and *L. brevis* (77). The precise role of these homologues should be investigated by the construction of targeted multiple isogenic *bsh*-mutant strains and *bsh* overexpression strains.

Bsh activity appears to be typical for inhabitants of the gastro-intestinal tract. A strong correlation has been found between the habitat of a specific bacterial species or strain and Bsh activity (107). This finding suggests a particular advantage of the capability to deconjugate bile salts for survival or persistence of bacteria under gastro-intestinal conditions. For this reason, the capability to hydrolyse bile salts has often been included as a prerequisite in the selection of probiotic bacteria. However, the precise advantage that a bacterium would gain from the expression of Bsh activity is unclear. For example, it has been suggested that Bsh activity plays a role in the detoxification of bile. E.g., in *Lactobacillus amylovorus*, *L. plantarum* and *L. monocytogenes*, the level of Bsh activity was found to correlate to sensitivity to bile salts (11, 31, 33, 37, 49). This correlation may be confirmed by the fact that Bsh activity is rare amongst Gram-negative bacteria, which are believed to be inherently more resistant to bile salts, although little is known about the exact mechanism. In fact, enrichment media for Gram-negative bacteria commonly contain bile salts. However, other groups reported that bile salt tolerance in *Lactobacillus spp.* is

not related to the level (2) or presence (86) of bile salt hydrolase activity. Since most Bsh enzymes show a preference for glycine-conjugated bile salts as compared to taurine-conjugated bile salts (e.g., in *Clostridium*, *Bifidobacterium*, and *Lactobacillus* (28, 67, 108, 113)), the lack of correlation found in the latter reports may be due to the use of taurine-conjugated bile salts to detect Bsh activity. Furthermore, the contribution of Bsh activity to bile tolerance can possibly only be assessed properly when comparing isogenic *bsh*-mutant strains, since membrane characteristics greatly influence bile tolerance (for review, see (11)). For example, damage to cell membrane lipopolysaccharides by freezing of *Escherichia coli* was shown to increase susceptibility to bile salts (26). Furthermore, *L. plantarum* was shown to regulate membrane synthesis in response to bile salt stress (22). The wide-spread preference of Bshs for glycine-conjugated bile salts may reinforce the hypothesis that bile salt hydrolase activity serves to detoxify bile salts, since glycine-conjugated bile salts are very toxic to bacterial cells, whereas taurine-conjugated bile salts appear to be less toxic. Finally, deconjugated bile salts precipitate more readily, leading to their removal from the environment and excretion in faeces.

Another commonly encountered hypothesis for the role of Bsh activity in bacteria is that the glycine and taurine liberated by deconjugation of bile salts may be used as carbon, nitrogen and energy source, thus conferring an advantage for bile salt-hydrolyzing strains in the competitive environment of the intestine (e.g., in *Clostridium* and *Bifidobacterium* (60, 108, 119)). However, experiments in lactobacilli showed that none of the strains tested were able to utilize either the amino acid or steroid moiety of the bile salts (45, 110).

Importance of lactobacilli. Especially lactobacilli were found to be of importance for *in vivo* bile salt deconjugation in the small intestine (18, 110, 111). For example, in mice that harboured a complex intestinal microbiota, but not lactobacilli, bile salt hydrolase activity in ileal contents was found to be reduced 90 % as compared to *Lactobacillus*-reconstituted mice (110). Using the same mouse model, the bile salt hydrolase activity produced by the lactobacilli was shown to be relevant *in vivo* for bile salt deconjugation levels in the small intestine (i.e., 70 % and 20 % of bile salts deconjugated for lactobacillus-reconstituted and *Lactobacillus*-free animals, respectively).

In addition, there is an increasing interest in the use of lactobacilli as a probiotic to positively influence host physiology and health status. The key notion in the use of probiotics is that any harmful effects of some of the resident bacteria can be, at least temporarily, balanced by shifting of composition of the gut microbiota towards more beneficial organisms. Especially lactobacilli and bifidobacteria have been considered to provide specific health benefits to the host, such as lowering of gas production, immunostimulation, antitumor activity, and the production of short chain fatty acids (for review, see (50)). Indeed, specific *Lactobacillus* strains have been used as probiotics (58). Especially in infantile, antibiotic, and travellers' diarrhoea, *Lactobacillus* spp. were found to

reduce the incidence or duration of diarrhoea (for review, see (42). Furthermore, *Lactobacillus* spp. were found to improve *Clostridium difficile* colitis, and increased resistance to food-borne pathogens (42). In addition, a probiotic mixture of four strains of lactobacilli, three strains of bifidobacteria, and one strain of *Streptococcus* called VSL#3 was found to improve inflammatory bowel diseases such as pouchitis (46, 47), and Crohn's disease (23).

The administration of large amounts of bile salt-hydrolysing probiotic bacteria could potentially result in a shift in intestinal bile salt composition that might be associated with an undesirable impact on intestinal physiology. Therefore, the influence of the delivery of bile salt hydrolase activity to the intestine on host response deserves attention. Considering their apparent importance in relation to host response, lactobacilli are excellent candidate organisms to study the functionality of Bsh in bacteria, the impact of Bsh activity of the microbiota, and of probiotics on host physiology.

Delivery of enzymes in the gastro-intestinal tract. The use of lactobacilli to influence host response does not need to be limited to the implementation of *wild-type* strains. For example, derivatives of *L. lactis* and *L. plantarum* have been used to deliver cytokines such as IL-10 and vaccines (e.g., tetanus toxin) to the intestinal tract of mice (21, 48, 57, 96, 102-104). The use of recombinant lactic acid bacteria appears to have a wide applicability, ranging from use in prevention of infectious diseases, allergies, inflammatory bowel diseases, and delivery of therapeutic proteins (54). Clearly, delivery of bile salt hydrolase by *wild-type* and genetically modified lactobacilli could prove to be an important tool in elucidating the influence of Bsh on host response. Indeed, the implementation of viable bacteria as a delivery vehicle is attractive, especially for use in the food industry, now that the use of fermented food products has become wide-spread among consumers. However, containment of genetically modified organisms used as probiotics remains subject to debate. For this purpose, self-containment strategies have been devised (104).

OUTLINE OF THIS THESIS

In this thesis, the functionality of bile salt hydrolases in Gram-positive bacteria, with a focus on *Lactobacillus plantarum* WCFS1, and the impact of delivery of bile salt hydrolase to the small intestine on the host were investigated.

Since bacterial bile salt hydrolase activity appears to play a significant role in both host intestinal physiology and bacterial survival and persistence in the intestinal tract, correct annotation of *bsh* genes in a particular bacterial species is of importance. Therefore, the annotation of *bsh* genes in Gram-positive bacteria was investigated in depth, leading to an improved annotation of eight conjugated bile acid (CBAH) superfamily members in various lactobacilli

and providing robust methodology for accurate annotation of this enzyme superfamily (**Chapter 2**).

In several intestinal lactobacilli, multiple genes coding for Bsh-family members have been predicted to be present in one organism. As a model organism, *L. plantarum* WCFS1 (68) was used, that was previously predicted to contain four *bsh*-like genes. The unravelling of the functionality of each of the separate genes requires the generation of multiple isogenic *bsh*-deletion strains. Therefore, a Cre-*lox*-based toolbox for the construction of multiple deletions and selectable-marker removal in Gram-positive organisms was designed and implemented in *L. plantarum* WCFS1 (**Chapter 3**).

Using the Cre-*lox* mutagenesis and existing overexpression tools, the functionality of the four Bsh-family members Bsh1, Bsh2, Bsh3, and Bsh4 of *L. plantarum* WCFS1 was investigated in **Chapter 4**. By a combination of heterologous over-expression and multiple *bsh*-deletion derivatives of *L. plantarum* WCFS1, Bsh1 was shown to be the major bile salt hydrolase in this strain, where it appeared to be involved in glycodeoxycholic acid tolerance. Although these experiments validated the prediction that *bsh2*, *bsh3*, and *bsh4* do not encode true Bsh enzymes (Chapter 2), they could also not entirely clarify the *in vivo* functionality of Bsh2, Bsh3, and Bsh4, which appear to encode enzymes with acylase activity possibly using penicillin-like chemicals as their preferred substrate.

In **Chapter 5**, the delivery of bile salt hydrolase activity to the small intestine using viable bacteria, with *L. plantarum* WCFS1 as a model organism, was compared to delivery using Bsh-whey protein/gum arabic microencapsulates in an *in vitro* model.

Finally, the influence of delivery of bile salt hydrolase activity by viable bacteria and whey protein/gum arabic microencapsulates on host response was investigated *in vivo* using a rat model (**Chapter 6**). However, no effect of delivery of Bsh on the intestinal bile salt composition or mucin excretion was detected. These results could indicate that the physiological relevance and magnitude of bile salt hydrolase activity of the natural microbiota and probiotics in the small intestine is of low significance.

REFERENCES

1. Agellon, L. B. 2002. p. 433-448. In D. E. a. V. Vance, J.E. (ed.), Biochemistry of Lipids, Lipoproteins and Membranes, 4th ed. Elsevier Science B.V.
2. Ahn, Y. T., Kim, G. B., Lim, K. S., Baek, Y. J. J. and Kim, H. U. 2003. Deconjugation of bile salts by *Lactobacillus acidophilus* isolates. Int. Dairy J. **13**:9.
3. Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin. 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. **85**:88-94.
4. Alrefai, W. A., and R. K. Gill. 2007. Bile acid transporters: structure, function, regulation and pathophysiological implications. Pharm. Res. **24**:1803-23.

5. **Amor, K. B., P. Breeuwer, P. Verbaarschot, F. M. Rombouts, A. D. Akkermans, W. M. De Vos, and T. Abee.** 2002. Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead bifidobacterium cells during bile salt stress. *Appl. Environ. Microbiol.* **68**:5209-16.
6. **Aslanzadeh, J., and L. J. Paulissen.** 1992. Role of type 1 and type 3 fimbriae on the adherence and pathogenesis of *Salmonella enteritidis* in mice. *Microbiol. Immunol.* **36**:351-9.
7. **Backhed, F., H. Ding, T. Wang, L. V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich, and J. I. Gordon.** 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl. Acad. Sci. USA* **101**:15718-23.
8. **Backhed, F., J. K. Manchester, C. F. Semenkovich, and J. I. Gordon.** 2007. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc. Natl. Acad. Sci. USA* **104**:979-84.
9. **Bateup, J. M., McConnell, M. A., Jenkinson, H. F., Tannock, G. W.** 1995. Comparison of *Lactobacillus* strains with respect to bile salt hydrolase activity, colonization of the gastrointestinal tract, and growth rate of the murine host. *Appl. Environ. Microbiol.* **61**:1147-1149.
10. **Batta, A. K., G. Salen, R. Arora, S. Shefer, M. Batta, and A. Person.** 1990. Side chain conjugation prevents bacterial 7-dehydroxylation of bile acids. *J. Biol. Chem.* **265**:10925-8.
11. **Begley, M., C. G. Gahan, and C. Hill.** 2005. The interaction between bacteria and bile. *FEMS Microbiol. Rev.* **29**:625-51.
12. **Begley, M., C. Hill, and C. G. Gahan.** 2006. Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol.* **72**:1729-38.
13. **Begley, M., R. D. Sleator, C. G. Gahan, and C. Hill.** 2005. Contribution of three bile-associated loci, *bsh*, *pva*, and *btlB*, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. *Infect. Immun.* **73**:894-904.
14. **Ben-Amor, K., H. Heilig, H. Smidt, E. E. Vaughan, T. Abee, and W. M. de Vos.** 2005. Genetic diversity of viable, injured, and dead fecal bacteria assessed by fluorescence-activated cell sorting and 16S rRNA gene analysis. *Appl. Environ. Microbiol.* **71**:4679-89.
15. **Berr, F., G. A. Kullak-Ublick, G. Paumgartner, W. Munzing, and P. B. Hylemon.** 1996. 7 alpha-dehydroxylating bacteria enhance deoxycholic acid input and cholesterol saturation of bile in patients with gallstones. *Gastroenterology* **111**:1611-20.
16. **Bhattacharjee, J. W., and B. S. Srivastava.** 1978. Mannose-sensitive haemagglutinins in adherence of *Vibrio cholerae* eltor to intestine. *J. Gen. Microbiol.* **107**:407-10.
17. **Bjorkhem, I., and S. Meaney.** 2004. Brain cholesterol: long secret life behind a barrier. *Arterioscler. Thromb. Vasc. Biol.* **24**:806-15.
18. **Bongaerts, G. P., R. S. Severijnen, A. Tangerman, A. Verrips, and J. J. Tolboom.** 2000. Bile acid deconjugation by *Lactobacilli* and its effects in patients with a short small bowel. *J. Gastroenterol.* **35**:801-4.
19. **Booijink, C. C., E. G. Zoetendal, M. Kleerebezem, and W. M. de Vos.** 2007. Microbial communities in the human small intestine: coupling diversity to metagenomics. *Future Microbiol.* **2**:285-95.
20. **Bron, P. A.** 2004. The molecular response of *Lactobacillus plantarum* to intestinal passage and conditions. Wageningen University, Wageningen.

21. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. *J. Bacteriol.* **186**:5721-9.
22. **Bron, P. A., D. Molenaar, W. M. de Vos, and M. Kleerebezem.** 2006. DNA microarray-based identification of bile-responsive genes in *Lactobacillus plantarum*. *J. Appl. Microbiol.* **100**:728-38.
23. **Campieri, M., and P. Gionchetti.** 1999. Probiotics in inflammatory bowel disease: new insight to pathogenesis or a possible therapeutic alternative? *Gastroenterology* **116**:1246-9.
24. **Carlstedt-Duke, B., T. Midtvedt, C. E. Nord, and B. E. Gustafsson.** 1986. Isolation and characterization of a mucin-degrading strain of *Peptostreptococcus* from rat intestinal tract. *Acta Pathol. Microbiol. Immunol. Scand.[B]* **94**:293-300.
25. **Cebra, J. J.** 1999. Influences of microbiota on intestinal immune system development. *Am. J. Clin. Nutr.* **69**:1046S-1051S.
26. **Chou, C. C., and S. J. Cheng.** 2000. Recovery of low-temperature stressed *E. coli* O157:H7 and its susceptibility to crystal violet, bile salt, sodium chloride and ethanol. *Int. J. Food Microbiol.* **61**:127-36.
27. **Christiaens, H., Leer, R. J., Pouwels, P. H., Verstraete, W.** 1992. Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* by using a direct plate assay. *Appl. Environ. Microbiol.* **58**:3792-3798.
28. **Coleman, J. P., and L. L. Hudson.** 1995. Cloning and characterization of a conjugated bile acid hydrolase gene from *Clostridium perfringens*. *Appl. Environ. Microbiol.* **61**:2514-20.
29. **Corr, S. C., Y. Li, C. U. Riedel, P. W. O'Toole, C. Hill, and C. G. Gahan.** 2007. Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. *Proc. Natl. Acad. Sci. USA* **104**:7617-21.
30. **Dawson, P. A., M. Hubbert, J. Haywood, A. L. Craddock, N. Zerangue, W. V. Christian, and N. Ballatori.** 2005. The heteromeric organic solute transporter alpha-beta, Ostalpha-Ostbeta, is an ileal basolateral bile acid transporter. *J. Biol. Chem.* **280**:6960-8.
31. **De Boever, P., and W. Verstraete.** 1999. Bile salt deconjugation by *Lactobacillus plantarum* 80 and its implication for bacterial toxicity. *J. Appl. Microbiol.* **87**:345-52.
32. **De Smet, I., P. De Boever, and W. Verstraete.** 1998. Cholesterol lowering in pigs through enhanced bacterial bile salt hydrolase activity. *Br. J. Nutr.* **79**:185-94.
33. **De Smet, I., L. Van Hoorde, M. Vande Woestyne, H. Christiaens, and W. Verstraete.** 1995. Significance of bile salt hydrolytic activities of lactobacilli. *J. Appl. Bacteriol.* **79**:292-301.
34. **De Vries, M. C.** 2006. Analyzing global gene expression of *Lactobacillus plantarum* in the human gastro-intestinal tract. Wageningen University, Wageningen.
35. **de Vries, M. C., E. E. Vaughan, M. Kleerebezem, and W. M. de Vos.** 2004. Optimising single cell activity assessment of *Lactobacillus plantarum* by fluorescent in situ hybridisation as affected by growth. *J Microbiol Methods* **59**:109-15.
36. **Derrien, M.** 2007. Mucin utilisation and host interactions of the novel intestinal microbe *Akkermansia muciniphila*. Wageningen University, Wageningen.
37. **Dussurget, O., D. Cabanes, P. Dehoux, M. Lecuit, C. Buchrieser, P. Glaser, and P. Cossart.** 2002. *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. *Mol. Microbiol.* **45**:1095-106.
38. **Elkins, C. A. a. S., D. C.** 1998. Identification of genes encoding conjugated bile salt hydrolase and transport in *Lactobacillus johnsonii* 100-100. *J. Bact.* **180**:4344-4349.

39. **Falk, P. G., L. V. Hooper, T. Midtvedt, and J. I. Gordon.** 1998. Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol. Mol. Biol. Rev.* **62**:1157-70.
40. **Feighner, S. D., and M. P. Dashkevich.** 1988. Effect of dietary carbohydrates on bacterial cholytaurine hydrolase in poultry intestinal homogenates. *Appl. Environ. Microbiol.* **54**:337-42.
41. **Feighner, S. D., and M. P. Dashkevich.** 1987. Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholytaurine hydrolase activity. *Appl. Environ. Microbiol.* **53**:331-6.
42. **Fooks, L. J., and G. R. Gibson.** 2002. Probiotics as modulators of the gut flora. *Br. J. Nutr.* **88 Suppl 1**:S39-49.
43. **Garcia-Diez, F., V. Garcia-Mediavilla, J. E. Bayon, and J. Gonzalez-Gallego.** 1996. Pectin feeding influences fecal bile acid excretion, hepatic bile acid and cholesterol synthesis and serum cholesterol in rats. *J. Nutr.* **126**:1766-71.
44. **Geyer, J., T. Wilke, and E. Petzinger.** 2006. The solute carrier family SLC10: more than a family of bile acid transporters regarding function and phylogenetic relationships. *Naunyn Schmiedebergs Arch. Pharmacol.* **372**:413-31.
45. **Gilliland, S. E. a. S., M. L.** 1977. Deconjugation of bile acids by intestinal lactobacilli. *Appl. Environ. Microbiol.* **33**:15-18.
46. **Gionchetti, P., F. Rizzello, A. Venturi, P. Brigidi, D. Matteuzzi, G. Bazzocchi, G. Poggioli, M. Miglioli, and M. Campieri.** 2000. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. *Gastroenterology* **119**:305-9.
47. **Gionchetti, P., F. Rizzello, A. Venturi, and M. Campieri.** 2000. Probiotics in infective diarrhoea and inflammatory bowel diseases. *J. Gastroenterol. Hepatol.* **15**:489-93.
48. **Grangette, C., S. Nutton, E. Palumbo, S. Morath, C. Hermann, J. Dewulf, B. Pot, T. Hartung, P. Hols, and A. Mercenier.** 2005. Enhanced antiinflammatory capacity of a *Lactobacillus plantarum* mutant synthesizing modified teichoic acids. *Proc. Natl. Acad. Sci. USA* **102**:10321-6.
49. **Grill, J. P., Perrin, S., Schneider, F.** 2000. Bile salt toxicity to some bifidobacteria strains: role of conjugated bile salt hydrolase and pH. *Can. J. Microbiol.* **46**:878-884.
50. **Guarner, F.** 2006. Enteric flora in health and disease. *Digestion* **73 Suppl 1**:5-12.
51. **Guban, J., D. R. Korver, G. E. Allison, and G. W. Tannock.** 2006. Relationship of dietary antimicrobial drug administration with broiler performance, decreased population levels of *Lactobacillus salivarius*, and reduced bile salt deconjugation in the ileum of broiler chickens. *Poult. Sci.* **85**:2186-94.
52. **Gustafsson, B. E., T. Midtvedt, and K. Strandberg.** 1970. Effects of microbial contamination on the cecum enlargement of germfree rats. *Scand. J. Gastroenterol.* **5**:309-14.
53. **Hagenbuch, B., and P. Dawson.** 2004. The sodium bile salt cotransport family SLC10. *Pflugers Arch.* **447**:566-70.
54. **Hanniffy, S., U. Wiedermann, A. Repa, A. Mercenier, C. Daniel, J. Fioramonti, H. Tlaskolova, H. Kozakova, H. Israelsen, S. Madsen, A. Vrang, P. Hols, J. Delcour, P. Bron, M. Kleerebezem, and J. Wells.** 2004. Potential and opportunities for use of recombinant lactic acid bacteria in human health. *Adv. Appl. Microbiol.* **56**:1-64.
55. **Hata, Y., S. Ota, T. Kawabe, A. Terano, M. Razandi, and K. J. Ivey.** 1994. Bile salts stimulate mucous glycoprotein secretion from cultured rabbit gastric mucosal cells. *J. Lab. Clin. Med.* **124**:395-400.

56. **Hofmann, A. F.** 1999. Bile Acids: The Good, the Bad, and the Ugly. *News Physiol. Sci.* **14**:24-29.
57. **Hols, P., P. Slos, P. Dutot, J. Reymund, P. Chabot, B. Delplace, J. Delcour, and A. Mercenier.** 1997. Efficient secretion of the model antigen M6-gp41E in *Lactobacillus plantarum* NCIMB 8826. *Microbiology* **143** (Pt 8):2733-41.
58. **Holzapfel, W. H., P. Haberer, J. Snel, U. Schillinger, and J. H. Huis in't Veld.** 1998. Overview of gut flora and probiotics. *Int. J. Food Microbiol.* **41**:85-101.
59. **Hooper, L. V., T. Midtvedt, and J. I. Gordon.** 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu. Rev. Nutr.* **22**:283-307.
60. **Huijghebaert, S. M., J. A. Mertens, and H. J. Eyssen.** 1982. Isolation of a bile salt sulfatase-producing *Clostridium* strain from rat intestinal microflora. *Appl. Environ. Microbiol.* **43**:185-92.
61. **Ide, T., and M. Horii.** 1989. Predominant conjugation with glycine of biliary and lumen bile acids in rats fed on pectin. *Br. J. Nutr.* **61**:545-57.
62. **Ide, T., M. Horii, K. Kawashima, and T. Yamamoto.** 1989. Bile acid conjugation and hepatic taurine concentration in rats fed on pectin. *Br. J. Nutr.* **62**:539-50.
63. **Ide, T., M. Horii, T. Yamamoto, and K. Kawashima.** 1990. Contrasting effects of water-soluble and water-insoluble dietary fibers on bile acid conjugation and taurine metabolism in the rat. *Lipids* **25**:335-40.
64. **Ide, T., and M. Sugano.** 1991. Interaction of dietary protein differing in sulfur amino acid content and pectin on bile acid conjugation in immature and mature rats. *J. Nutr.* **121**:985-93.
65. **Kararli, T. T.** 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm. Drug Dispos.* **16**:351-80.
66. **Kawamoto, K., I. Horibe, and K. Uchida.** 1989. Purification and characterization of a new hydrolase for conjugated bile acids, chenodeoxycholytaurine hydrolase, from *Bacteroides vulgatus*. *J. Biochem.* **106**:1049-53.
67. **Kim, G. B., C. M. Miyamoto, E. A. Meighen, and B. H. Lee.** 2004. Cloning and characterization of the bile salt hydrolase genes (*bsh*) from *Bifidobacterium bifidum* strains. *Appl. Environ. Microbiol.* **70**:5603-12.
68. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. USA* **100**:1990-5.
69. **Klinkspoor, J. H., R. Kuver, C. E. Savard, D. Oda, H. Azzouz, G. N. Tytgat, A. K. Groen, and S. P. Lee.** 1995. Model bile and bile salts accelerate mucin secretion by cultured dog gallbladder epithelial cells. *Gastroenterology* **109**:264-74.
70. **Klinkspoor, J. H., K. S. Mok, B. J. Van Klinken, G. N. Tytgat, S. P. Lee, and A. K. Groen.** 1999. Mucin secretion by the human colon cell line LS174T is regulated by bile salts. *Glycobiology* **9**:13-9.
71. **Klinkspoor, J. H., G. N. Tytgat, S. P. Lee, and A. K. Groen.** 1996. Mechanism of bile salt-induced mucin secretion by cultured dog gallbladder epithelial cells. *Biochem. J.* **316** (Pt 3):873-7.
72. **Krogfelt, K. A., H. Bergmans, and P. Klemm.** 1990. Direct evidence that the FimH protein is the mannose-specific adhesin of *Escherichia coli* type 1 fimbriae. *Infect. Immun.* **58**:1995-8.

73. **Kullak-Ublick, G. A., B. Stieger, and P. J. Meier.** 2004. Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology* **126**:322-42.
74. **Lambert, J. M., R. S. Bongers, and M. Kleerebezem.** 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **73**:1126-35.
75. **Leer, R. J., Christiaens, H., Verstraete, W., Peters, L., Posno, M., Pouwels, P. H.** 1993. Gene disruption in *Lactobacillus plantarum* strain 80 by site-specific recombination: isolation of a mutant strain deficient in conjugated bile salt hydrolase activity. *Mol. Gen. Genet.* **239**:269-272.
76. **Lin, M. C., W. Kramer, and F. A. Wilson.** 1990. Identification of cytosolic and microsomal bile acid-binding proteins in rat ileal enterocytes. *J. Biol. Chem.* **265**:14986-95.
77. **Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev, Y. Lou, D. Rohksar, S. Lucas, K. Huang, D. M. Goodstein, T. Hawkins, V. Plengvidhya, D. Welker, J. Hughes, Y. Goh, A. Benson, K. Baldwin, J. H. Lee, I. Diaz-Muniz, B. Dosti, V. Smeianov, W. Wechter, R. Barabote, G. Lorca, E. Altermann, R. Barrangou, B. Ganesan, Y. Xie, H. Rawsthorne, D. Tamir, C. Parker, F. Breidt, J. Broadbent, R. Hutkins, D. O'Sullivan, J. Steele, G. Unlu, M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, and D. Mills.** 2006. Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci. USA* **103**:15611-6.
78. **Marco, M. L., R. S. Bongers, W. M. de Vos, and M. Kleerebezem.** 2007. Spatial and temporal expression of *Lactobacillus plantarum* genes in the gastrointestinal tracts of mice. *Appl. Environ. Microbiol.* **73**:124-32.
79. **Marco, M. L., and M. Kleerebezem.** 2007. Assessment of real-time RT-PCR for quantification of *Lactobacillus plantarum* gene expression during stationary phase and nutrient starvation. *J. Appl. Microbiol.* (OnlineEarly Articles).
80. **Matsuo, K., H. Ota, T. Akamatsu, A. Sugiyama, and T. Katsuyama.** 1997. Histochemistry of the surface mucous gel layer of the human colon. *Gut* **40**:782-9.
81. **McAuliffe, O., R. J. Cano, and T. R. Klaenhammer.** 2005. Genetic analysis of two bile salt hydrolase activities in *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* **71**:4925-9.
82. **McGarr, S. E., J. M. Ridlon, and P. B. Hylemon.** 2005. Diet, anaerobic bacterial metabolism, and colon cancer: a review of the literature. *J. Clin. Gastroenterol.* **39**:98-109.
83. **Meier, P. J.** 1995. Molecular mechanisms of hepatic bile salt transport from sinusoidal blood into bile. *Am. J. Physiol.* **269**:G801-12.
84. **Meier, P. J., and B. Stieger.** 2002. Bile salt transporters. *Annu. Rev. Physiol.* **64**:635-61.
85. **Molin, G., B. Jeppsson, M. L. Johansson, S. Ahrne, S. Nobaek, M. Stahl, and S. Bengmark.** 1993. Numerical taxonomy of *Lactobacillus* spp. associated with healthy and diseased mucosa of the human intestines. *J. Appl. Bacteriol.* **74**:314-23.
86. **Moser, S. A. a. S., D. C.** 2001. Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. *Appl. Environ. Microbiol.* **67**:3476-3480.
87. **Nguyen, T. D., J. H. Kang, and M. S. Lee.** 2007. Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. *Int. J. Food Microbiol.* **113**:358-61.

88. **Oda, H., S. Kuroki, H. Yamashita, and F. Nakayama.** 1990. Effects of bile acid feeding on hepatic deoxycholate 7 alpha-hydroxylase activity in the hamster. *Lipids* **25**:706-10.
89. **Olsson, A., and M. Uhlen.** 1986. Sequencing and heterologous expression of the gene encoding penicillin V amidase from *Bacillus sphaericus*. *Gene* **45**:175-81.
90. **Pereira, D. I., and G. R. Gibson.** 2002. Effects of consumption of probiotics and prebiotics on serum lipid levels in humans. *Crit. Rev. Biochem. Mol. Biol.* **37**:259-81.
91. **Pereira, D. I., A. L. McCartney, and G. R. Gibson.** 2003. An in vitro study of the probiotic potential of a bile-salt-hydrolyzing *Lactobacillus fermentum* strain, and determination of its cholesterol-lowering properties. *Appl. Environ. Microbiol.* **69**:4743-52.
92. **Rajilic-Stojanovic, M.** 2007. Diversity of the human gastrointestinal microbiota - novel perspectives from high throughput analyses. Wageningen University, Wageningen.
93. **Rathinaswamy, P., A. V. Pundle, A. A. Prabhune, H. Sivaraman, J. A. Brannigan, G. G. Dodson, and C. G. Suresh.** 2005. Cloning, purification, crystallization and preliminary structural studies of penicillin V acylase from *Bacillus subtilis*. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **61**:680-3.
94. **Rehfeld, J. F.** 2004. Clinical endocrinology and metabolism. Cholecystokinin. *Best. Pract. Res. Clin. Endocrinol. Metab.* **18**:569-86.
95. **Ridlon, J. M., D. J. Kang, and P. B. Hylemon.** 2006. Bile salt biotransformations by human intestinal bacteria. *J. Lipid Res.* **47**:241-59.
96. **Robinson, K., L. M. Chamberlain, K. M. Schofield, J. M. Wells, and R. W. Le Page.** 1997. Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nat. Biotechnol.* **15**:653-7.
97. **Russell, D. W., and K. D. Setchell.** 1992. Bile acid biosynthesis. *Biochemistry* **31**:4737-49.
98. **Shanahan, F.** 2001. Inflammatory bowel disease: immunodiagnostics, immunotherapeutics, and ecotherapeutics. *Gastroenterology* **120**:622-35.
99. **Shekels, L. L., C. T. Lyftogt, and S. B. Ho.** 1996. Bile acid-induced alterations of mucin production in differentiated human colon cancer cell lines. *Int. J. Biochem. Cell. Biol.* **28**:193-201.
100. **Shimotoyodome, A., S. Meguro, T. Hase, I. Tokimitsu, and T. Sakata.** 2000. Decreased colonic mucus in rats with loperamide-induced constipation. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **126**:203-12.
101. **Smith, E. A., and G. T. Macfarlane.** 1996. Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. *J. Appl. Bacteriol.* **81**:288-302.
102. **Steidler, L.** 2002. In situ delivery of cytokines by genetically engineered *Lactococcus lactis*. *Antonie Van Leeuwenhoek* **82**:323-31.
103. **Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut.** 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* **289**:1352-5.
104. **Steidler, L., and P. Rottiers.** 2006. Therapeutic drug delivery by genetically modified *Lactococcus lactis*. *Ann. N. Y. Acad. Sci.* **1072**:176-86.
105. **Stellwag, E. J., Hylemon, P.B.** 1976. Purification and characterization of bile salt hydrolase from *Bacteroides fragilis* subsp. *fragilis*. *Biochim. Biophys. Acta* **452**:165-76.

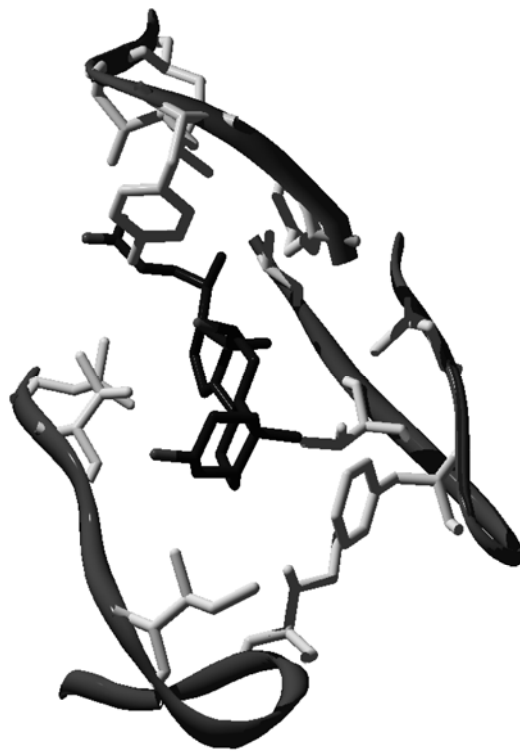
106. **Suchy, F. J., and M. Ananthanarayanan.** 2006. Bile salt excretory pump: biology and pathobiology. *J. Pediatr. Gastroenterol. Nutr.* **43 Suppl 1**:S10-6.
107. **Tanaka, H., Doesburg, K., Iwasaki, T., Mierau, I.** 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. *J. Dairy Sci.* **82**:2530-2535.
108. **Tanaka, H., Hashiba, H., Kok, J., Mierau, I.** 2000. Bile salt hydrolase of *Bifidobacterium longum* - biochemical and genetic characterization. *Appl. Environ. Microbiol.* **66**:2502-2512.
109. **Tannock, G. W.** 2001. Molecular assessment of intestinal microflora. *Am. J. Clin. Nutr.* **73**:410S-414S.
110. **Tannock, G. W., M. P. Dashkevich, and S. D. Feighner.** 1989. Lactobacilli and bile salt hydrolase in the murine intestinal tract. *Appl. Environ. Microbiol.* **55**:1848-51.
111. **Tannock, G. W., A. Tangerman, A. Van Schaik, and M. A. McConnell.** 1994. Deconjugation of bile acids by lactobacilli in the mouse small bowel. *Appl. Environ. Microbiol.* **60**:3419-20.
112. **Taranto, M. P., F. Sesma, A. Pesce de Ruiz Holgado and G.F. de Valdez.** 1997. Bile salt hydrolase plays a key role on cholesterol removal by *Lactobacillus reuteri*. *Biotech. Lett.* **19**:3.
113. **Taranto, M. P., F. Sesma, G. Font de Valdez.** 1999. Localization and primary characterization of bile salt hydrolase from *Lactobacillus reuteri*. *Biotech. Lett.* **21**:4.
114. **Thoma-Uszynski, S., S. Stenger, O. Takeuchi, M. T. Ochoa, M. Engele, P. A. Sieling, P. F. Barnes, M. Rollinghoff, P. L. Bolcskei, M. Wagner, S. Akira, M. V. Norgard, J. T. Belisle, P. J. Godowski, B. R. Bloom, and R. L. Modlin.** 2001. Induction of direct antimicrobial activity through mammalian toll-like receptors. *Science* **291**:1544-7.
115. **Trauner, M., and J. L. Boyer.** 2003. Bile salt transporters: molecular characterization, function, and regulation. *Physiol. Rev.* **83**:633-71.
116. **Underhill, D. M., and A. Ozinsky.** 2002. Toll-like receptors: key mediators of microbe detection. *Curr. Opin. Immunol.* **14**:103-10.
117. **Une, M., and T. Hoshita.** 1994. Natural occurrence and chemical synthesis of bile alcohols, higher bile acids, and short side chain bile acids. *Hiroshima J. Med. Sci.* **43**:37-67.
118. **Valeur, N., P. Engel, N. Carbajal, E. Connolly, and K. Ladefoged.** 2004. Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract. *Appl. Environ. Microbiol.* **70**:1176-81.
119. **Van Eldere, J., J. Robben, G. De Pauw, R. Merckx, and H. Eyssen.** 1988. Isolation and identification of intestinal steroid-desulfating bacteria from rats and humans. *Appl. Environ. Microbiol.* **54**:2112-7.
120. **van Kooyk, Y., and T. B. Geijtenbeek.** 2003. DC-SIGN: escape mechanism for pathogens. *Nat. Rev. Immunol.* **3**:697-709.
121. **Walters, H. C., A. L. Craddock, H. Fusegawa, M. C. Willingham, and P. A. Dawson.** 2000. Expression, transport properties, and chromosomal location of organic anion transporter subtype 3. *Am. J. Physiol. Gastrointest. Liver Physiol.* **279**:G1188-200.
122. **Weis, W. I., M. E. Taylor, and K. Drickamer.** 1998. The C-type lectin superfamily in the immune system. *Immunol. Rev.* **163**:19-34.
123. **Wijaya, A., A. Hermann, H. Abriouel, I. Specht, N. M. Yousif, W. H. Holzapfel, and C. M. Franz.** 2004. Cloning of the bile salt hydrolase (*bsh*) gene from *Enterococcus faecium* FAIR-E 345 and chromosomal location of *bsh* genes in food enterococci. *J. Food Prot.* **67**:2772-8.

Introduction

124. **Wold, A. E., M. Thorssen, S. Hull, and C. S. Eden.** 1988. Attachment of *Escherichia coli* via mannose- or Gal alpha 1----4 Gal beta-containing receptors to human colonic epithelial cells. *Infect. Immun.* **56**:2531-7.
125. **Zoetendal, E. G., B. Cheng, S. Koike, and R. I. Mackie.** 2004. Molecular microbial ecology of the gastrointestinal tract: from phylogeny to function. *Curr. Issues Intest. Microbiol.* **5**:31-47.
126. **Zoetendal, E. G., E. E. Vaughan, and W. M. de Vos.** 2006. A microbial world within us. *Mol. Microbiol.* **59**:1639-50.

Chapter 2.

Improved annotation of conjugated bile acid hydrolase superfamily members in Gram-positive bacteria.



J. M. Lambert
R. J. Siezen
W. M. de Vos
M. Kleerebezem

2. Improved annotation of conjugated bile acid hydrolase superfamily members in Gram-positive bacteria

Most Gram-positive bacteria inhabiting the gastro-intestinal tract are capable of hydrolyzing bile salts. Bile salt hydrolysis is supposed to play an important role in various biological processes in the host. Therefore, correct annotation in public databases of bile salt hydrolases (Bsh; EC3.5.1.24) in bacteria is of importance, especially for lactobacilli, which are considered to play a major role in bile salt hydrolysis *in vivo*. In the present study, all enzymes in public databases belonging to the bile salt hydrolase family and closely related penicillin V acylase (Pva; 3.5.1.11) family were compared, with the sequences annotated as Bsh in *Lactobacillus plantarum* WCFS1 as an example. In Gram-positive bacteria, a clear distinction could be made between the two families using sequence alignment, phylogenetic clustering, and protein homology modelling. Biochemical and structural data on experimentally verified Bsh and Pva enzymes were used for validation of function prediction. Hidden-Markov models were constructed from the sequence alignments that enable a more accurate prediction of Bsh-encoding genes and their distinction from the Pva family. Many Pva-related sequences appeared to be annotated incorrectly as Bsh in public databases. This refinement in the annotation of Bsh family-members especially influences the prediction of the function of *bsh*-like genes in species of the genus *Lactobacillus*, which is discussed in detail.

INTRODUCTION

Bile salts play an important role in lipid digestion in mammals. In the liver, bile salts are synthesized from cholesterol and conjugated with the amino acids glycine or taurine. Following excretion into the intestinal lumen, the amino acid part of the bile salts can be hydrolysed by bile salt hydrolases (Bshs; EC3.5.1.24), also designated choloylglycine hydrolase or conjugated bile acid hydrolase, produced by the intestinal microbiota.

Bacterial Bsh activity has received much attention based on its postulated role in various biological processes in the host. For example, bile salt hydrolysis may be involved in serum cholesterol lowering (21). Bile salt deconjugation is the gate-keeping reaction in further oxidation and dehydroxylation steps of bile salts by intestinal bacteria, which includes the production of secondary bile salts that have been linked to various intestinal diseases such as the formation of gallstones and colon cancer (24). Moreover, *in vitro* studies have suggested that bile salt deconjugation plays a role in the mucin production and excretion in the intestinal lumen (14), which could affect the nutritional environment encountered by the intestinal microbiota and intestinal transit time (26).

Based on the biological implications of Bsh activity, it is important to provide a correct annotation of *bsh* genes in bacterial DNA sequences. Notably,

Bsh amino acid sequences resemble those of penicillin V acylases (Pva, EC3.5.1.11) and belong to the same enzyme superfamily of linear amide C-N hydrolases (Pfam CBAH, PF02275). Although both Bsh and Pva are capable of hydrolysing the same type of chemical bond, the overall chemical nature of their substrates is quite different (bile salts and penicillins, respectively; Figure 1). Especially, the steroid moiety of bile salts is significantly more voluminous when compared to the corresponding moiety of penicillin V. Penicillin acylase-encoding genes may at times be incorrectly annotated as bile salt hydrolase, as was found for example in *Listeria monocytogenes* (3), leading to unreliable prediction of Bsh activity presence in a particular bacterial strain.

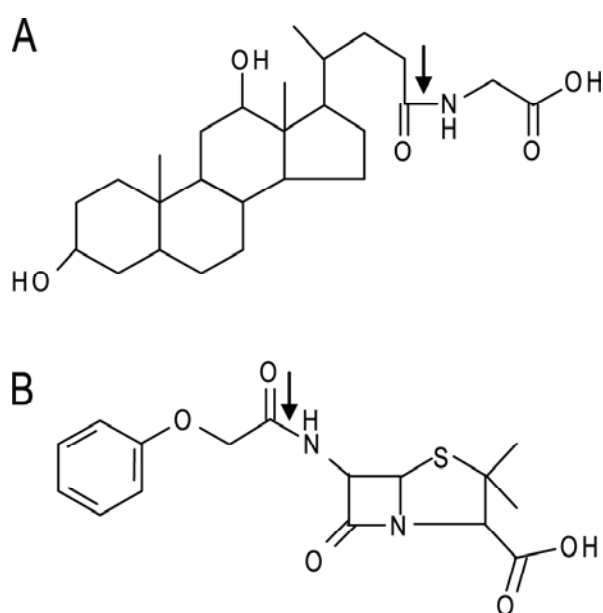


Figure 1. Chemical structure of bile salts and penicillins. As an example, the bile salt glycodeoxycholic acid (panel A) and penicillin V (panel B) are shown. The bond that is hydrolysed by either Bsh or Pva is indicated by an arrow.

To confidently draw conclusions from *in silico* analysis of CBAH superfamily members, information on experimentally established enzyme activity and/or structure of Bsh and Pva enzymes is indispensable (Table 1). For example, experimentally verified Bsh enzymes can be found for *Enterococcus faecium* (33), *Bifidobacterium* species (11, 29), *Clostridium* (12), *Lactobacillus* species (5, 10, 16, 17) and *Listeria monocytogenes* (9). Experimentally verified Pva-family proteins can be found for *Bacillus* species (19, 23) and *Listeria monocytogenes* (3).

Among bacterial species that are capable of bile salt hydrolysis, especially lactobacilli have been reported to play a major role in bile salt deconjugation *in vivo* (4, 7, 22, 31). In *Lactobacillus*-free mice, the Bsh activity in the ileal content was found to be reduced by almost 90 % when compared to *Lactobacillus*-reconstituted mice (30). Moreover, one of the commonly used criteria in selection of candidate probiotic strains is their ability to hydrolyse bile salts (2). There is a growing interest in the use of bile salt-hydrolysing

Lactobacillus strains in fermented milk products for lowering of serum cholesterol concentrations (21). Consequently, the accuracy of annotation of *bsh* genes in lactobacilli deserves special attention.

In this study, a combination of *in silico* methods was employed to distinguish accurately between the Bsh and Pva family members. All protein sequences of CBAH superfamily members from public databases were compared and their functionality was predicted using phylogenetic profiling, sequence alignment and 3D-modeling techniques, incorporating experimentally verified Bsh and Pva proteins. In addition, hidden Markov models were constructed that enabled the distinction between Bsh and Pva proteins. Previous annotation of Bsh-family members in several *Lactobacillus* species was based mainly on sequence homology, and was found to be mostly incorrect, as exemplified for the four CBAH superfamily paralogs found in the model organism *Lactobacillus plantarum* WCFS1 (13, 16). In this work, we propose a refined annotation of Bsh and Pva family members in lactobacilli.

Table 1. Experimentally verified Bsh and Pva family proteins.

Sequence ID nr ¹	Bacterial strain	Reference	Remarks
Bsh family			
Q6R974	<i>B. bifidum</i> ATCC11863	(11)	
Q9KK62	<i>B. longum</i> SBT2928	(29)	Crystallised (15)
P54965	<i>C. perfringens</i> 13	(6)	Crystallised (25)
Q83YZ2	<i>E. faecium</i> FAIRE-E 345	(33)	
Q9F660	<i>L. johnsonii</i> 100-100	(10)	α subunit
P97038	<i>L. johnsonii</i> 100-100	(10)	β subunit
Q8Y5J3	<i>L. monocytogenes</i> EGDe	(9)	
S51638 (GB)	<i>L. plantarum</i> LP80	(5)	
Q06115	<i>L. plantarum</i> WCFS1	(16)	Bsh1
Pva family			
P12256	<i>B. sphaericus</i> ATCC14577	(19)	Crystallised (27)
Q2HPP6	<i>B. subtilis</i> NCIMB	(23)	Crystallised
Q8Y9S7	<i>L. monocytogenes</i> EGDe	(3)	

¹Sequence ID as used in the SwissProt database.

GB; Genbank database

MATERIALS AND METHODS

Sequence alignment, phylogenetic profiling and construction of hidden Markov models. For phylogenetic profiling of Bsh- and Pva-family members, the sequences of putative CBAH superfamily members were retrieved from the ERGO database (20) using BLASTP (1) and as seed the sequence of the experimentally verified bile salt hydrolase (Bsh1, lp_3536) of *L. plantarum* WCFS1 (13, 16). Multiple sequence alignments were created with MUSCLE (Edgar, 2004). Clustal X (Thompson, 1997) was used to display multiple

sequence alignments and to create phylogenetic trees, and these trees were then displayed with LOFT (32). Hidden Markov models (HMMs) were constructed from the multiple sequence alignments using HMMER 2.3.2 (8).

3D-structure homology modelling. The 3-D structures used as templates for homology modelling were bile salt hydrolase (Bsh) from *Bifidobacterium longum* (2hez.pdb), Bsh from *Clostridium perfringens* in complex with reaction products taurine and deoxycholate (2bjf.pdb), and penicillin V acylase (Pva) in complex with diethane diol from *Bacillus sphaericus* (2pva.pdb). Homology modelling was performed with the ‘The Whatif/Yasara Twinset’ software (www.yasara.com).

RESULTS

Phylogenetic profiling. The sequences of the CBAH superfamily members (retrieved using the sequence of the experimentally verified Bsh1 of *L. plantarum* WCFS1 (16) as seed) were analyzed together with sequences of experimentally studied Bsh and Pva enzymes that were not present in the ERGO database (20) (Table 1). The superfamily tree shows that sequences derived from gram-negative and Gram-positive organisms were clearly divided in two separate groups.

Focusing on the group of sequences derived from Gram-positives, two clusters could be identified (Figure 2). The first cluster, hereafter called the Bsh-cluster, consisted of sequences that appeared phylogenetically closely related, and contained all biochemically verified Bsh proteins, including Bsh1 of *L. plantarum* WCFS1. The Bsh-cluster contained twenty-five sequences, of which twenty-three were annotated in the public NCBI database (<http://ncbi.nlm.nih.gov>) as Bsh and one was annotated as penicillin acylase (Table 2). For one sequence, no function was annotated; however, this protein was experimentally shown to be a Bsh (9). Furthermore, this cluster contained ten sequences derived from lactobacilli, of which nine sequences were annotated as Bsh, and one sequence was annotated as Pva. The second cluster, hereafter called the Pva-cluster, consisted of sequences that generally appeared to be less tightly related phylogenetically as compared to those in the Bsh-cluster. All experimentally verified Pva proteins were encompassed within the Pva cluster. This cluster contained forty-nine sequences, of which eleven were annotated as penicillin acylase, thirty were annotated as bile salt hydrolase, one was annotated as an ABC transporter, and seven sequences lacked a functional annotation (Table 2). Furthermore, eight sequences of lactobacilli were grouped in the Pva-cluster, of which seven were annotated as Bsh and one was annotated as Pva. The three paralogous proteins previously annotated as Bsh (Bsh2, Bsh3 and Bsh4; (13)) that are predicted to be present in addition to the experimentally verified Bsh1 (16) in *L. plantarum* WCFS1 were grouped phylogenetically in the Pva-cluster, with Bsh2 and Bsh3 being more closely related than Bsh4.

Importantly, all experimentally verified Bsh and Pva enzymes were grouped correctly during *in silico* analysis, strongly suggesting that Bsh- and Pva-proteins can be distinguished properly using the phylogenetic profiling technique.

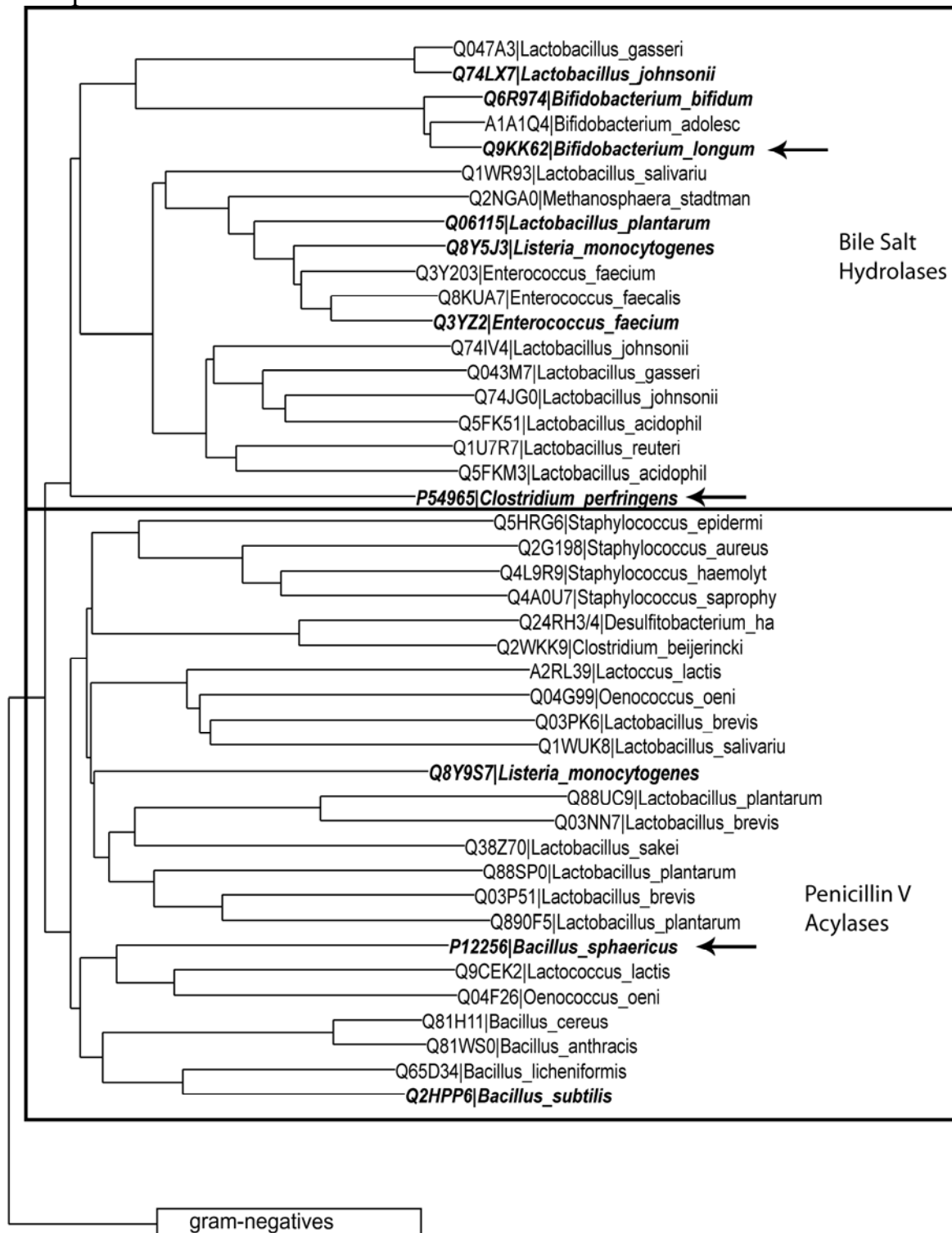


Figure 2. Family tree of Bsh- and Pva-family members in Gram-positive bacteria. For clarity, one representative sequence is shown when sequences of different strains of the same organism are identical or nearly identical. Sequences shown in italics were experimentally verified, and sequences with known 3D-structures were marked with arrows.

Improved annotation of conjugated bile acid hydrolase superfamily members

Table 2. Annotation of Gram-positive CBAH superfamily members in the phylogenetic tree.

Sequence ID number ¹	Species	Exp. verified	Current annotation
<u>Bsh-cluster</u>			
Q8KUA7	<i>Enterococcus faecalis</i> V583		bile salt hydrolase
Q83YZ2	<i>Enterococcus faecium</i> FAIR-E345	+	bile salt hydrolase
Q8Y5J3	<i>Listeria monocytogenes</i> EGD-e	+	<i>hypothetical protein</i>
Q71XU7	<i>Listeria monocytogenes</i> 4b F2365		bile salt hydrolase
Q3Y203	<i>Enterococcus faecium</i> DO		bile salt hydrolase
Q2NGA0	<i>Methanosphaera stadtmanae</i> DSM3091		bile salt hydrolase
Q1WR93	<i>Lactobacillus salivarius</i> UCC118		bile salt hydrolase
Q5FK51	<i>Lactobacillus acidophilus</i> NCFM		bile salt hydrolase
Q1U7R7	<i>Lactobacillus reuteri</i> 100-23		bile salt hydrolase ²
Q9F660	<i>Lactobacillus johnsonii</i> 100-100	+	bile salt hydrolase
P97038	<i>Lactobacillus johnsonii</i> 100-100	+	bile salt hydrolase
Q74IV4	<i>Lactobacillus johnsonii</i> NCC 533		bile salt hydrolase
Q047A3	<i>Lactobacillus gasseri</i> ATCC 33323		bile salt hydrolase
Q74LX7	<i>Lactobacillus johnsonii</i> NCC 533		bile salt hydrolase
Q5FKM3	<i>Lactobacillus acidophilus</i> NCFM		bile salt hydrolase
Q043M7	<i>Lactobacillus gasseri</i> ATCC 33323		<i>penicillin acylase</i>
Q74JG0	<i>Lactobacillus johnsonii</i> NCC 533		bile salt hydrolase
S51638 (GB)	<i>Lactobacillus plantarum</i> LP80	+	bile salt hydrolase
Q06115	<i>Lactobacillus plantarum</i> WCFS1 (Bsh1)	+	bile salt hydrolase
Q6R974	<i>Bifidobacterium bifido</i> ATCC11863	+	bile salt hydrolase
A1A1Q4	<i>Bifidobacterium adolescentis</i> ATCC 15703		bile salt hydrolase
Q9KK62	<i>Bifidobacterium longum</i> SBT2928	+	bile salt hydrolase
Q8G653	<i>Bifidobacterium longum</i> NCC2705		bile salt hydrolase
Q0TT83	<i>Clostridium perfringens</i> ATCC 13124		bile salt hydrolase
P54965	<i>Clostridium perfringens</i> 13	+	bile salt hydrolase
<u>Pva-cluster</u>			
Q03PK6	<i>Lactobacillus brevis</i> ATCC 367		penicillin acylase
Q1WUK8	<i>Lactobacillus salivarius</i> UCC118		<i>bile salt hydrolase</i>
A2RL39	<i>Lactococcus lactis</i> MG1363		penicillin acylase
Q04G99	<i>Oenococcus oeni</i> PSU-1		penicillin acylase
Q03NN7	<i>Lactobacillus brevis</i> ATCC 367		<i>bile salt hydrolase</i>
Q38Z70	<i>Lactobacillus sakei</i> 23K		<i>bile salt hydrolase</i>
Q03P51	<i>Lactobacillus brevis</i> ATCC 367		<i>bile salt hydrolase</i>
Q890F5	<i>Lactobacillus plantarum</i> WCFS1 (Bsh2)		<i>bile salt hydrolase</i>
Q88SP0	<i>Lactobacillus plantarum</i> WCFS1 (Bsh3)		<i>bile salt hydrolase</i>
Q88UC9	<i>Lactobacillus plantarum</i> WCFS1 (Bsh4)		<i>bile salt hydrolase</i>
Q1Y4P3	<i>Staphylococcus aureus</i> JH9		<i>bile salt hydrolase</i> ²
Q2YV99	<i>Staphylococcus aureus</i> RF122		<i>bile salt hydrolase</i>
Q8NYG2	<i>Staphylococcus aureus</i> MW2		<i>hypothetical protein</i>
Q2FJZ5	<i>Staphylococcus aureus</i> USA300		<i>bile salt hydrolase</i>
Q5HJA0	<i>Staphylococcus aureus</i> COL		<i>bile salt hydrolase</i>

Improved annotation of conjugated bile acid hydrolase superfamily members

Table 2 continued

Sequence number ¹	ID	Species	Exp. verified	Current annotation
Q99WVI		<i>Staphylococcus aureus</i> Mu50		penicillin acylase
Q6GK36		<i>Staphylococcus aureus aureus</i> MRSA252		<i>bile salt hydrolase</i>
Q2G198		<i>Staphylococcus aureus</i> NCTC 8325		<i>hypothetical protein</i>
Q7A7T1		<i>Staphylococcus aureus</i> N315		<i>hypothetical protein</i>
Q6GCJ7		<i>Staphylococcus aureus aureus</i> MSSA476		<i>bile salt hydrolase</i>
Q4L9R9		<i>Staphylococcus haemolyticus</i> JCSC1435		<i>hypothetical protein</i>
Q4A0U7		<i>Staphylococcus saprophyticus</i> ATCC15305		<i>bile salt hydrolase</i>
Q8CQ66		<i>Staphylococcus epidermidis</i> ATCC 12228		penicillin acylase
Q5HRG6		<i>Staphylococcus epidermidis</i> RP62A		penicillin acylase
Q18WR1		<i>Desulfitobacterium hafniense</i> DCB-2		<i>bile salt hydrolase</i> ²
Q24RH3		<i>Desulfitobacterium hafniense</i> Y51		<i>hypothetical protein</i>
Q2WKK9		<i>Clostridium beijerincki</i> NCIMB-8052		<i>bile salt hydrolase</i> ²
Q9CEK2		<i>Lactococcus lactis</i> IL1403		penicillin acylase
Q9CGG1		<i>Lactococcus lactis</i> IL1403		penicillin acylase
Q04F26		<i>Oenococcus oeni</i> PSU-1		<i>bile salt hydrolase</i>
Q04G99		<i>Oenococcus oeni</i> PSU-1		penicillin acylase
P12256		<i>Bacillus sphaericus</i> ATCC14577	+	penicillin acylase
Q8Y9S7		<i>Listeria monocytogenes</i> EGD-e	+	<i>hypothetical protein</i>
Q65D34		<i>Bacillus licheniformis</i> ATCC 14580 XX		<i>bile salt hydrolase</i>
Q2HHP6		<i>Bacillus subtilis</i> 168	+	penicillin acylase
Q81H11		<i>Bacillus cereus</i> ATCC14579		<i>bile salt hydrolase</i>
A0RHD9		<i>Bacillus thuringiensis</i> Al Hakam		<i>bile salt hydrolase</i>
Q2AKF7		<i>Bacillus-weihenstephaniensis</i> KBAB4		<i>bile salt hydrolase</i> ²
Q6HF48		<i>Bacillus thuringiensis</i> konkukian		<i>ABC transporter, permease</i>
Q732V9		<i>Bacillus cereus</i> ATCC 10987		<i>bile salt hydrolase</i>
Q81WS0*		<i>Bacillus anthracis</i> A2012		<i>bile salt hydrolase</i> ²
Q81WS0		<i>Bacillus anthracis</i> Ames 0581		<i>bile salt hydrolase</i>
Q81WS0		<i>Bacillus anthracis</i> Sterne		<i>bile salt hydrolase</i>
Q81WS0		<i>Bacillus anthracis</i> Ames		<i>bile salt hydrolase</i>
Q81WS0		<i>Bacillus anthracis</i> KrugerB		<i>bile salt hydrolase</i> ²
Q81WS0		<i>Bacillus anthracis</i> A1055		<i>hypothetical protein</i>
Q81WS0		<i>Bacillus anthracis</i> CNEVA 9066		<i>bile salt hydrolase</i> ²
Q81WS0		<i>Bacillus anthracis</i> Australia 94		<i>bile salt hydrolase</i> ²
Q81WS0		<i>Bacillus anthracis</i> Vollum		<i>bile salt hydrolase</i> ²

¹Sequence ID as derived from the SwissProt database; ²When no annotation was provided in the NCBI public database (<http://ncbi.nlm.nih.gov>), the annotation (when provided) from the ERGO database (<http://wit.integratedgenomics.com>) was used; ID, identification; Exp., experimentally; GB, GenBank database. Annotations that were predicted to be incorrect are shown in bold italics.

* All *B. anthracis* amino acid sequences are identical, and not distinguished by SwissProt.

3D protein homology modelling and sequence alignment. The 3D-structures of the crystallized Bsh of *Bifidobacterium longum* (15)(2HEZ.pdb), Bsh of *Clostridium perfringens* (25)(2bjf.pdb), and Pva of *Bacillus sphaericus* (27) (2PVA.pdb) were superimposed, and the catalytic residues and binding pockets were compared (Figure 3A and 3B). Based on multiple sequence alignment, the catalytic and binding pocket residues of all Bsh and Pva family members were defined. The residues present in the substrate binding pocket appeared to be more conserved in the Bsh-cluster compared to the Pva-cluster. Catalytic and putative substrate binding residues of the Bsh- and Pva-cluster, and of the four *L. plantarum* enzymes are compared in Table 3.

Table 3. Catalytic and binding pocket residues in Bsh-family members. (A) Bsh-cluster members; (B) Pva-cluster members. For active site residues, the first number designates the residue number in the template 3D structure used in homology modelling (*B. longum* Bsh and *B. sphaericus* Pva) (Figure 3), whereas the number between brackets designates the residue number as used in Clustal X multiple sequence alignment.

A.

Residue	<i>B. longum</i>	<i>C. perfringens</i>	Bsh1 <i>L. plantarum</i>	In family	Remarks
<u>Catalytic</u>					
2	C	C	C	C	Conserved
18	R	R	R	R	Conserved
21	D	D	D	D	Conserved
82	N	N	N	N	<i>L. gasseri</i> : S
177	N	N	N	N	Conserved
229	R	R	R	R	Conserved
<u>Binding pocket</u>					
<i>Loop 1</i>					
20	L	M	F	L, F	All very hydrophobic
22	W	I	Y	Y, L, W	All hydrophobic
24	F	Y	I	I, F	Mostly hydrophobic, but variable
26	Y	F	Y	Y, F	All hydrophobic
<i>Loop 2</i>					
58	G	G	T	G, A, S	Neutral to hydrophobic
59	V	T	A	A, V, I	All hydrophobic
61	M	F	V	M, V	Mostly hydrophobic, but variable
66	M	T	L	L	Mostly hydrophobic
68	F	A	Y	F, Y	All hydrophobic
103	F	Y	F	F, Y	All hydrophobic
<i>Loop 3</i>					
133	deletion	I	F	F, I	All hydrophobic
137	Q	I	L	L	All hydrophobic
140	S	T	S	S, A	Neutral to hydrophobic
142	L	L	L	L	Conserved; very hydrophobic

B.

Residue	<i>B. sphæricus</i>	Bsh2 <i>Lp</i> ¹	Bsh3 <i>Lp</i> ¹	Bsh4 <i>Lp</i> ¹	In family	Remarks
<u>Catalytic</u>						
1	C	C	C	C	C	Conserved
17	R	R	R	R	R	Conserved
20	D	D	D	D	D	Conserved
82	Y	Y	Y	Y	Y	Occasionally T
175	N	N	N	N	N	Except <i>D. hafniense</i> (Q)
228	R	R	R	R	R	Conserved
<u>Binding pocket</u>						
<i>Loop 1</i>						
19	M	M	M	M	M	Conserved; very hydrophobic
21	F	F	F	F	F, W	All hydrophobic
23	M	V	F	T	F, Y	Mostly hydrophobic, but variable
25	P	F	L	T	L, F, M	Mostly hydrophobic, but variable
<i>Loop 2</i>						
58	G	G	G	G	G	Conserved
59	S	R	R	R	R	Very variable
61	D	L	L	H	L, M	Very variable
66	V	I	I	L	I, L, D	Variable
68	Y	T	V	A	A, F, G	Neutral to hydrophobic
<i>Loop 3</i>						
135	I	L	L	D	L, D, T, I	Variable
136	L	I	L	T	L, I, T	Mostly hydrophobic, but variable
140	P	P	V	Y	P, A, T	Variable
142	L	L	L	F	L	All very hydrophobic

¹*Lp*: *Lactobacillus plantarum*

In Figure 3A, the substrate binding pocket of *Bifidobacterium longum* Bsh is shown, with a deoxycholate molecule (as an example of the steroid moiety of bile salts) modeled in an orientation as in the *C. perfringens* 3D structure (2bjf.pdb). The side chains lining this pocket are shown to come mainly from three surface loops, and are predominantly hydrophobic. The substrate binding pocket is also hydrophobic in other sequences from the Bsh-cluster (Table 3), which is in agreement with the hydrophobic nature of the steroid moiety of bile salts. As expected, the predicted catalytic and substrate binding residues of the experimentally verified Bsh1 enzyme of *L. plantarum* WCFS1 are most similar to those of the *B. longum* and *C. perfringens* bile salt hydrolases, as shown in Table 3 and Figure 3C.

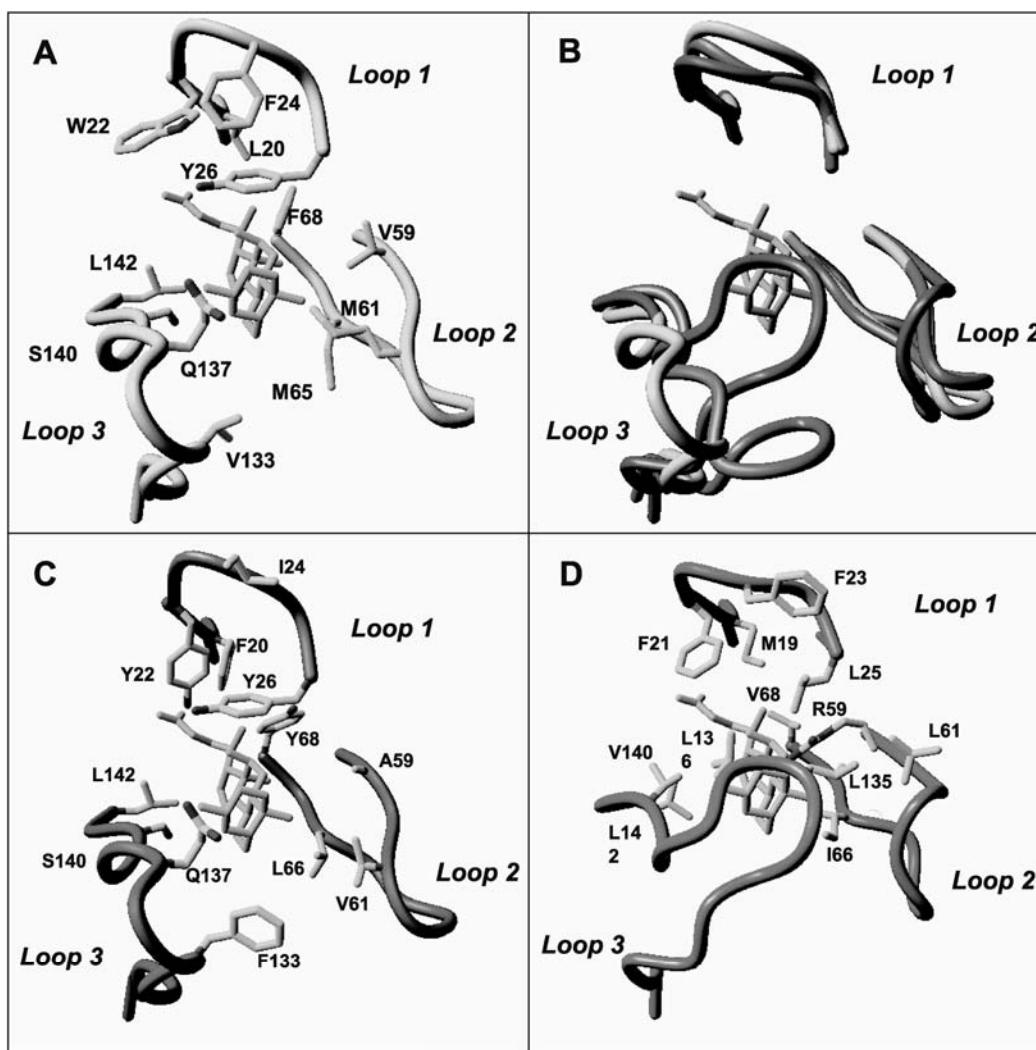


Figure 3. Three-dimensional structure comparison of Bsh and Pva proteins. (A) Substrate binding pocket of Bsh of *B. longum* (2hez.pdb), showing only side chains of three loops (light grey) presumed to be relevant in binding substrates such as deoxycholate, which is modelled in an orientation as found in the 3D structure of *C. perfringens* (2bjf.pdb). (B) Main chain backbone superposition of the three loops of the substrate binding pockets of Bsh of *B. longum* (light grey), *C. perfringens* (medium grey) and Pva of *B. sphaericus* (dark grey). (C) Substrate binding pocket of Bsh1 of *L. plantarum* WCFS1 as predicted by sequence alignment, using the 3D structure of the binding pocket of Bsh of *B. longum* as a template. (D) Substrate binding pocket of Bsh2 of *L. plantarum* WCFS1 as predicted by sequence alignment, using the 3D structure of the binding pocket of Pva of *B. sphaericus* as a template.

Clear differences were found in the 3D-structure of the substrate binding pockets of proteins of the Bsh- and Pva-clusters. Firstly, loop 3 differs in length and orientation, as illustrated in Figure 3B. In Pva of *B. sphaericus* loop 3 is folded inward, severely reducing the size of the binding pocket. Secondly, the side chains of loops 2 and 3 lining the binding pocket are far more variable in size and hydrophilicity/hydrophobicity in the Pva group (Table 3). The catalytic residues, however, appeared to be highly conserved in both respective groups. Five out of the six catalytic residues appeared to be identical in both the Bsh-

and Pva-cluster, whereas the catalytic residue at position 82 in the 3D-model usually was asparagine in the Bsh-cluster, and tyrosine or occasionally threonine (four out of twenty-seven) in the Pva-cluster. Predicted binding pocket residues of Bsh2 and Bsh3 of *L. plantarum* WCFS1 appeared to resemble those of the 3D-structure of Pva as illustrated in Table 3 and Figure 3D, whereas the residues of Bsh4 appeared to fit less well to that of either Bsh of *B. longum* or Pva of *C. perfringens*. Thus, using 3D-modeling, the separation of Bsh-family members in either the Bsh- or Pva-cluster as found by phylogenetic profiling was confirmed based on 3D models of the substrate binding pocket and consequently, the ability to accommodate bile salt molecules.

Hidden Markov models for distinction between Bsh and Pva family members. From the Clustal X alignments, separate and distinctive hidden Markov models (HMMs) were constructed for the proteins of the Bsh- and Pva-clusters. Using the Bsh-HMM to annotate the CBAH superfamily members, only proteins of the Bsh-cluster were found as best hit. Likewise, only proteins of the Pva-cluster were found as best hit using the Pva-HMM. Subsequently, the two HMMs were used for searching all publicly available sequenced genomes. Using the Bsh-HMM, all best hits found in the sequenced genomes were already present in the Bsh-cluster of the phylogenetic tree (Figure 2), and no additional hits were found (E-value score better than $1.80 \cdot 10^{-179}$). Furthermore, with the Bsh-HMM, a clear separation was found in the E-value score of the members of the Bsh-cluster (E score better than $1.80 \cdot 10^{-179}$) and subsequent hits (E-value worse than $2.00 \cdot 10^{-108}$), which were members of the Pva-cluster. Likewise, using the Pva-HMM, all best hits (E-value score better than $6.60 \cdot 10^{-132}$) found in the sequenced genomes consisted of members of the Pva-cluster. Again, a clear separation was found in the E-value score of the Pva sequences (E-value better than $6.60 \cdot 10^{-132}$) and subsequent hits (E-value worse than $8.20 \cdot 10^{-97}$), which were either members of the Bsh-cluster or gram-negative sequences not present in our phylogenetic tree. These findings strongly suggest that the HMMs presented here enable an accurate prediction of the functionality of any CBAH superfamily member, classifying them either as Bsh enzymes (EC3.5.1.24) or Pva-related enzymes (EC3.5.1.11).

DISCUSSION

In this study, we strived to improve the annotation of CBAH superfamily members by employing both phylogenetic clustering and 3D-modeling techniques in addition to the more conventional method of sequence alignment. The *in silico* analyses employed here consistently distinguished between the Bshs and the homologous penicillin V acylase enzymes. The Bsh enzymes are considered especially relevant for microbes that reside in the mammalian

intestinal system, where the lactobacilli are considered to be among the most important participants in bile salt deconjugation *in vivo* (30).

The construction of a phylogenetic tree is an excellent way of visualising the relatedness of sequences that is more informative than the study of sequence homology alone. In our study, phylogenetic clustering clearly separated Gram-positive CBAH superfamily members into two groups: a Bsh-cluster, that is predicted to contain Bshs, and a Pva-cluster, where its members are predicted to be penicillin-acylase-related proteins. Interestingly, it appeared that all strains that were represented only in the Bsh-cluster are typical gut-related bacteria (i.e. Bifidobacteria, *Enterococcus faecalis* and *faecium*, and *Lactobacillus acidophilus* species), while strains represented only in the Pva-cluster were not. This finding is in good agreement with the previously reported correlation between the presence of Bsh activity in lactic acid bacteria and isolation from the intestine or feces (28). The prediction of the functionality of the CBAH superfamily members by phylogenetic profiling was reinforced by the fact that the functionality of all experimentally verified Bsh and Pva proteins matched the *in silico* clustering.

Our results indicate that the sequences annotated as Bsh in the Pva-cluster are likely to be incorrectly annotated in the public NCBI and KEGG databases (<http://ncbi.nlm.nih.gov>, <http://www.genome.jp/kegg>), and probably are Pva or Pva-related enzymes. Particularly for *Lactobacillus* species, the refinement of the annotation of functionality of this family of enzymes could have an important impact on their anticipated influence on gastrointestinal bile salt metabolism and its cognate physiological consequences in the host. According to our current analysis, the present Pva annotation of one the *L. gasseri* ATCC 33323 CBAH superfamily sequences (SwissProt ID Q047A3) should be corrected to Bsh, while the sequences in the Pva-cluster currently annotated as Bsh in *L. salivarius* UCC118 (SwissProt ID Q1WUK8), *L. brevis* ATCC 367 (SwissProt ID Q03NN7 and Q03P51), *L. sakei* 23K (SwissProt ID Q38Z70), and *L. plantarum* WCFS1 (SwissProt ID Q890F5 [Bsh2]; Q88SP0 [Bsh3], and Q88UC9 [Bsh4]) should be corrected to Pva-related (Table 2).

Furthermore, the members of the Pva- and Bsh-clusters were not only found to be phylogenetically, but also structurally different. 3D-modeling and sequence alignment showed clear differences in the 3D-structure of the substrate binding pockets of Bsh- and Pva-enzymes, as illustrated in Figure 3. Especially, one loop of the substrate binding pocket (loop 3) in Pva is folded inwards as compared to the Bsh enzymes. As a consequence, it is unlikely that bile salts can be accommodated in the substrate binding pocket of Pva (Figure 1). The members of the Pva-cluster were found to be less related than members of the Bsh-cluster during phylogenetic analysis. This finding was reflected in a higher variability in the residues of the binding pocket of members of the Pva-cluster as compared to members of the Bsh-cluster, suggesting considerable variation in putative penicillin derivatives or similar molecules that could be bound. As

expected, the predicted substrate binding pocket of the experimentally verified bile salt hydrolase Bsh1 of *L. plantarum* (16) structurally resembled the Bsh enzymes of *B. longum* (15) and *C. perfringens* (25). In addition, the paralogs Bsh2, Bsh3, and Bsh4 were clustered in the Pva-group during phylogenetic analysis, with Bsh2 and Bsh3 being more related to each other than to Bsh4. This is in agreement with the finding that the predicted substrate binding pocket of Bsh2 and Bsh3 appeared to structurally resemble the Pva enzyme of *B. sphaericus* (27) considerably more than that of Bsh4. Possibly, Bsh4 encodes an enzyme related to Pva, capable of acylating a substrate other than penicillin V.

Notably, one of the catalytic residues appeared to differ between the Bsh- and Pva-cluster. This finding facilitates accurate prediction of the functionality of the Bsh-family members as Bsh or Pva (-related) from their amino acid sequences. Novel and discriminative HMMs were constructed to distinguish between Bsh and Pva-family members. Thereby, our analyses defined the discriminative characteristics of the Pva- and Bsh-enzyme families, which appeared not feasible using the available conserved motif databases. For example, the conserved domain search tool (18) provided by NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) aims at detection and prediction of conserved domains in proteins, and includes domains imported from SMART, Pfam or COG databases. However, this tool attributes ‘penicillin V acylase, also known as conjugated bile salt acid hydrolase’ to the best conserved domain found in all experimentally verified Bsh- and Pva-proteins used here (Table 1), and thereby fails to distinguish between Bsh- and penicillin acylase-like domains. Analogously, neither SMART, Pfam nor COG databases provide a correct discriminative domain description for all experimentally verified Bsh- and Pva-proteins (data not shown).

In conclusion, we have provided evidence that various Pva-related sequences are wrongly annotated as Bsh in various Gram-positive bacteria, including several lactobacilli. Especially for intestinal lactobacilli, the capacity to deconjugate bile acids has been suggested to be of importance for their survival capacity in the gastrointestinal tract of mammals and is proposed to strongly influence gut physiology. Therefore, appropriate annotation of the Bsh enzymes in *Lactobacillus* and other lactic acid bacteria genomes is important to predict their *in situ* functionality in the gastrointestinal tract. The methodology presented here provides the *in silico* tools to effectively distinguish the genes encoding the important Bsh enzymes from those encoding closely related Pva-like activities.

ACKNOWLEDGEMENTS

The authors would like to thank Bernadet Renckens and Michiel Wels for their technical support and valuable contribution to this manuscript.

REFERENCES

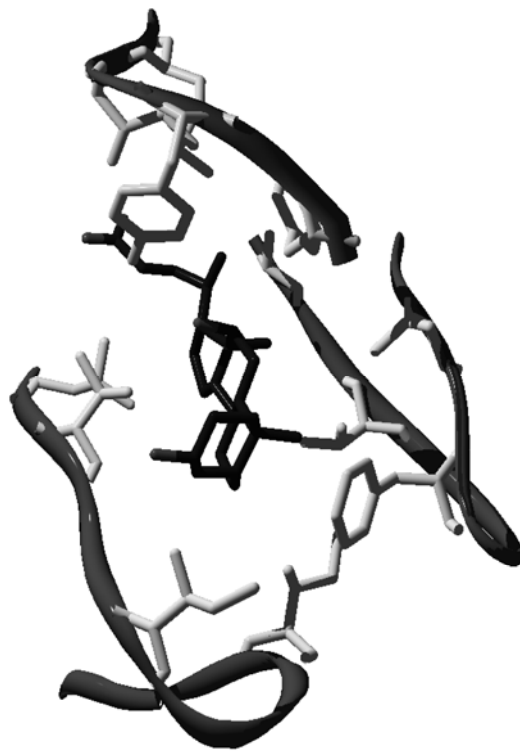
1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403-10.
2. **Begley, M., C. Hill, and C. G. Gahan.** 2006. Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol.* **72**:1729-38.
3. **Begley, M., R. D. Sleator, C. G. Gahan, and C. Hill.** 2005. Contribution of three bile-associated loci, *bsh*, *pva*, and *btlB*, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. *Infect. Immun.* **73**:894-904.
4. **Bongaerts, G. P., R. S. Severijnen, A. Tangerman, A. Verrips, and J. J. Tolboom.** 2000. Bile acid deconjugation by *Lactobacilli* and its effects in patients with a short small bowel. *J. Gastroenterol.* **35**:801-4.
5. **Christiaens, H., Leer, R. J., Pouwels, P. H., Verstraete, W.** 1992. Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* by using a direct plate assay. *Appl. Environ. Microbiol.* **58**:3792-3798.
6. **Coleman, J. P., and L. L. Hudson.** 1995. Cloning and characterization of a conjugated bile acid hydrolase gene from *Clostridium perfringens*. *Appl. Environ. Microbiol.* **61**:2514-20.
7. **De Smet, I., L. Van Hoorde, M. Vande Woestyne, H. Christiaens, and W. Verstraete.** 1995. Significance of bile salt hydrolytic activities of lactobacilli. *J. Appl. Bacteriol.* **79**:292-301.
8. **Durbin, R., S.R. Eddy, A. Krogh, and G. Mitchison.** 1998. Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids. Cambridge University Press, Cambridge, UK.
9. **Dussurget, O., D. Cabanes, P. Dehoux, M. Lecuit, C. Buchrieser, P. Glaser, and P. Cossart.** 2002. *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. *Mol. Microbiol.* **45**:1095-106.
10. **Elkins, C. A. a. S., D. C.** 1998. Identification of genes encoding conjugated bile salt hydrolase and transport in *Lactobacillus johnsonii* 100-100. *J. Bact.* **180**:4344-4349.
11. **Kim, G. B., C. M. Miyamoto, E. A. Meighen, and B. H. Lee.** 2004. Cloning and characterization of the bile salt hydrolase genes (*bsh*) from *Bifidobacterium bifidum* strains. *Appl. Environ. Microbiol.* **70**:5603-12.
12. **Kirby, L. C., Klein, R. A., Coleman, J. P.** 1995. Continuous spectrophotometric assay of conjugated bile acid hydrolase. *Lipids* **30**:863-867.
13. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. USA* **100**:1990-5.
14. **Klinkspoor, J. H., K. S. Mok, B. J. Van Klinken, G. N. Tytgat, S. P. Lee, and A. K. Groen.** 1999. Mucin secretion by the human colon cell line LS174T is regulated by bile salts. *Glycobiology* **9**:13-9.
15. **Kumar, R. S., J. A. Brannigan, A. A. Prabhune, A. V. Pundle, G. G. Dodson, E. J. Dodson, and C. G. Suresh.** 2006. Structural and functional analysis of a conjugated bile salt hydrolase from *Bifidobacterium longum* reveals an evolutionary relationship with penicillin V acylase. *J. Biol. Chem.* **281**:32516-25.
16. **Lambert, J. M., R. S. Bongers, and M. Kleerebezem.** 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **73**:1126-35.

17. **Leer, R. J., Christiaens, H., Verstraete, W., Peters, L., Posno, M., Pouwels, P. H.** 1993. Gene disruption in *Lactobacillus plantarum* strain 80 by site-specific recombination: isolation of a mutant strain deficient in conjugated bile salt hydrolase activity. *Mol. Gen. Genet.* **239**:269-272.
18. **Marchler-Bauer, A., J. B. Anderson, P. F. Cherukuri, C. DeWeese-Scott, L. Y. Geer, M. Gwadz, S. He, D. I. Hurwitz, J. D. Jackson, Z. Ke, C. J. Lanczycki, C. A. Liebert, C. Liu, F. Lu, G. H. Marchler, M. Mullokandov, B. A. Shoemaker, V. Simonyan, J. S. Song, P. A. Thiessen, R. A. Yamashita, J. J. Yin, D. Zhang, and S. H. Bryant.** 2005. CDD: a Conserved Domain Database for protein classification. *Nucleic Acids Res.* **33**:D192-6.
19. **Olsson, A., and M. Uhlen.** 1986. Sequencing and heterologous expression of the gene encoding penicillin V amidase from *Bacillus sphaericus*. *Gene* **45**:175-81.
20. **Overbeek, R., N. Larsen, T. Walunas, M. D'Souza, G. Pusch, E. Selkov, Jr., K. Liolios, V. Joukov, D. Kaznadzey, I. Anderson, A. Bhattacharyya, H. Burd, W. Gardner, P. Hanke, V. Kapatral, N. Mikhailova, O. Vasieva, A. Osterman, V. Vonstein, M. Fonstein, N. Ivanova, and N. Kyrpides.** 2003. The ERGO genome analysis and discovery system. *Nucleic Acids Res.* **31**:164-71.
21. **Pereira, D. I., and G. R. Gibson.** 2002. Effects of consumption of probiotics and prebiotics on serum lipid levels in humans. *Crit. Rev. Biochem. Mol. Biol.* **37**:259-81.
22. **Pereira, D. I., A. L. McCartney, and G. R. Gibson.** 2003. An in vitro study of the probiotic potential of a bile-salt-hydrolyzing *Lactobacillus fermentum* strain, and determination of its cholesterol-lowering properties. *Appl. Environ. Microbiol.* **69**:4743-52.
23. **Rathinaswamy, P., A. V. Pundle, A. A. Prabhune, H. Sivaraman, J. A. Brannigan, G. G. Dodson, and C. G. Suresh.** 2005. Cloning, purification, crystallization and preliminary structural studies of penicillin V acylase from *Bacillus subtilis*. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **61**:680-3.
24. **Ridlon, J. M., D. J. Kang, and P. B. Hylemon.** 2006. Bile salt biotransformations by human intestinal bacteria. *J. Lipid Res.* **47**:241-59.
25. **Rossocha, M., R. Schultz-Heienbrok, H. von Moeller, J. P. Coleman, and W. Saenger.** 2005. Conjugated bile acid hydrolase is a tetrameric N-terminal thiol hydrolase with specific recognition of its cholyl but not of its tauryl product. *Biochemistry* **44**:5739-48.
26. **Shimotoyodome, A., S. Meguro, T. Hase, I. Tokimitsu, and T. Sakata.** 2000. Decreased colonic mucus in rats with loperamide-induced constipation. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **126**:203-12.
27. **Suresh, C. G., A. V. Pundle, H. SivaRaman, K. N. Rao, J. A. Brannigan, C. E. McVey, C. S. Verma, Z. Dauter, E. J. Dodson, and G. G. Dodson.** 1999. Penicillin V acylase crystal structure reveals new Ntn-hydrolase family members. *Nat. Struct. Biol.* **6**:414-6.
28. **Tanaka, H., Doesburg, K., Iwasaki, T., Mierau, I.** 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. *J. Dairy Sci.* **82**:2530-2535.
29. **Tanaka, H., Hashiba, H., Kok, J., Mierau, I.** 2000. Bile salt hydrolase of *Bifidobacterium longum* - biochemical and genetic characterization. *Appl. Environ. Microbiol.* **66**:2502-2512.
30. **Tannock, G. W., M. P. Dashkevich, and S. D. Feighner.** 1989. Lactobacilli and bile salt hydrolase in the murine intestinal tract. *Appl. Environ. Microbiol.* **55**:1848-51.
31. **Tannock, G. W., A. Tagerman, A. Van Schaik, and M. A. McConnell.** 1994. Deconjugation of bile acids by lactobacilli in the mouse small bowel. *Appl. Environ. Microbiol.* **60**:3419-20.

32. **van der Heijden, R. T., B. Snel, V. van Noort, and M. A. Huynen.** 2007. Orthology prediction at scalable resolution by phylogenetic tree analysis. *BMC Bioinformatics* **8**:83.
33. **Wijaya, A., A. Hermann, H. Abriouel, I. Specht, N. M. Yousif, W. H. Holzapfel, and C. M. Franz.** 2004. Cloning of the bile salt hydrolase (*bsh*) gene from *Enterococcus faecium* FAIR-E 345 and chromosomal location of *bsh* genes in food enterococci. *J. Food Prot.* **67**:2772-8.

Chapter 3.

A Cre-*lox*-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*.



J. M. Lambert
R. S. Bongers
M. Kleerebezem

3. A Cre-*lox*-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*

The classic strategy to achieve gene deletion variants is based on double cross-over integration of non-replicating vectors into the genome. In addition, recombination systems such as Cre-*lox* have been used extensively mainly in eukaryotic organisms. This study presents the construction of a Cre-*lox*-based system for multiple gene deletions in *Lactobacillus plantarum* that could be adapted for use in Gram-positive bacteria. First, an effective mutagenesis vector (pNZ5319) was constructed that allows direct cloning of blunt-end PCR products representing homologous recombination target regions. Using this mutagenesis vector, double cross-over gene replacement mutants could be readily selected based on their antibiotic resistance phenotype. In the resulting mutants, the target gene is replaced by a *lox66*-P₃₂-*cat*-*lox71* cassette, where *lox66* and *lox71* are mutant variants of *loxP* and P₃₂-*cat* is a chloramphenicol resistance cassette. The *lox* sites serve as recognition sites for the Cre enzyme, which is a protein that belongs to the integrase family of site-specific recombinases. Thus, transient Cre recombinase expression in double-cross over mutants leads to recombination of the *lox66*-P₃₂-*cat*-*lox71* cassette into a double mutant *loxP* site, called *lox72*, which displays strongly reduced recognition by Cre. The effectiveness of the Cre-*lox*-based strategy for multiple gene deletions was demonstrated by construction of both single and double gene deletions at the *mela* and *bshI* loci on the chromosome of the Gram-positive model organism *Lactobacillus plantarum* WCFS1. Furthermore, the efficiency of the Cre-*lox*-based system in multiple gene replacements was determined by successive mutagenesis of the genetically closely linked loci *mela* and *lacS2* in *L. plantarum* WCFS1. The fact that 99.4 % of the clones that were analysed had undergone correct Cre-*lox* resolution emphasizes the suitability of the system described here for multiple gene replacement and deletion strategies in a single genetic background.

INTRODUCTION

The development of tools for genetic engineering in Gram-positive bacteria is highly valuable for research applications. The classic strategy to obtain gene deletion variants is based on homologous recombination, using double cross-over integration of heterologous non-replicating vectors such as pUC (34, 36, 37, 42) , pACYC184 (6, 48, 50), or their derivatives in the genome . Several convenient systems that derive from this strategy use conditionally replicating vectors, such as the thermo-sensitive pG⁺host system (40), and the RepA dependent lactococcal pORI system (originating from pWV01) (12, 33, 51) and its broad host range derivative (42, 51).

In addition to systems that derive from the classic strategy, various site-specific recombination systems such as Flp-*FRT* (52), Gateway (Invitrogen), ParA-*res* (31), TnpR-*res* (13) and Cre-*lox* are used in mutational strategies. However, these systems have until now not been available for construction of an unlimited number of mutations in the same genetic background in Gram-positive bacteria. For this purpose, the versatile Cre-*lox* system is a promising candidate. The Cre recombinase is a 38 kD protein that belongs to the integrase family of site-specific recombinases. It catalyses co-factor independent recombination between two of its recognition sites, called *loxP*. The 34 bp consensus for *loxP* sites consists of an asymmetrical core spacer of 8 bp, defining the orientation of the *loxP* site, and two 13 bp palindromic flanking sequences (1, 23). A DNA sequence that is flanked by *loxP* sites is excised when the *loxP* sites are convergently oriented, whereas the sequence is inverted when the *loxP* sites are divergently oriented. Cre recombinase is able to act on both inter- and intramolecular *loxP* sites, although recombination of intramolecular *lox* sites is kinetically favorable (32).

The versatile properties of Cre recombinase make it ideal for use in many genetic manipulation strategies. Therefore, the Cre-*lox* system has been used in a wide variety of eukaryotic such as plants (20), yeast (54), mice (45, 55), feline cell lines (30), human cell lines (26, 43), and chicken cell lines (5). For example, recombination of intermolecular *loxP* sites has been used for site-specific integration of transfected DNA into the chromosome (4, 29, 30). Many strategies use recombination of *loxP* sites to excise the intermediate DNA sequence. This includes work on conditional gene deletions (5) and recombinatorial activation of gene expression (55). In particular, an important application of the Cre-*lox* system is selectable marker excision in gene replacements. Commonly used gene replacement strategies result in the introduction of a selectable marker into the genome, facilitating selection of gene mutations that might cause growth retardation. However, the expression of the marker may result in polar effects on expression of up- and downstream located genes. Selectable-marker removal from the genome by Cre-*lox* recombination is an elegant and efficient way of circumventing this issue and has therefore been used frequently in for example plants (20), mouse cell lines (2), and yeast (22).

Notably, use of native *loxP* sites for consecutive rounds of gene replacement and subsequent selectable-marker removal would lead to the integration of multiple *loxP* sites in the genome that can still be recognized by Cre. To minimize genetic instability, *lox* sites containing mutations within the inverted repeats (*lox66* and *lox71*; Figure 1) have been used in plants (4) and chicken cell lines (5). Recombination of *lox66* and *lox71* results in a *lox72* site that shows strongly reduced binding affinity for Cre, allowing for repeated gene deletion in a single genetic background.



Figure 1. Schematic representation of mutant *lox66* and *lox71* sites, which after Cre recombination result in a double mutant *lox72* site. Sequences in bold type are mutated compared to the native *loxP* site (shown as reference).

In contrast to the many eukaryote examples, the Cre-*lox* system has been used much less frequently in prokaryotic organisms. For example, in *Escherichia coli*, mechanistic studies on Cre recombination were performed (3, 44). In *Lactobacillus plantarum*, the Cre-*lox* system was used for conditional gene deletions in the murine GI-tract (10). In addition, Cre-*lox* mediated selectable-marker removal in gene replacements has been used in the Gram-negative bacteria *Methylobacterium extorquens*, *Burkholderia fungorum*, *Escherichia coli*, and *Pseudomonas aeruginosa* (41, 46, 49). However, these experiments implemented native *loxP* sites for multiple gene replacements. Previously, it was shown in *Lactococcus lactis*, *Corynebacterium glutamicum* and *Salmonella typhimurium* that Cre readily excises or inverts large fragments of DNA flanked by *loxP* sites in prokaryotes (14, 15, 57, 62), thereby leading to genomic instability.

Here we describe the construction of a Cre-*lox*-based toolbox for multiple gene deletions in a single genetic background in Gram-positive bacteria, which combines the advantages of selectable gene replacement and a marker-free, in-frame gene deletion in the final strain. For this purpose, a mutagenesis vector was constructed and used for classic double cross-over replacement of target genes by the selectable marker cassette *lox66*-P₃₂-*cat*-*lox71*, which can be recombined from the chromosome into a double-mutant *lox72* site by transient Cre recombinase expression from a second plasmid.

To validate the effectiveness of the system described here, the mutagenesis targets *mela*, *bsh1*, and *lacS2* were selected in the model organism *L. plantarum* WCFS1, of which the genome was sequenced (28). The *mela* and *bsh1* genes are genetically unlinked loci, whereas the *mela* and *lacS2* loci are genetically closely linked. Although only experimentally tested in this bacterium, the simple, functional implementation of the same basic characteristics involved in this host to other bacterial hosts will be discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids and primers. The bacterial strains, plasmids and primers used in this study and their relevant features are listed in Table 1 and 2.

As a model strain for Gram-positive bacteria, *L. plantarum* WCFS1 (28) was used. *L. plantarum* was grown at 37°C in MRS broth (Difco, West Molesey, United Kingdom) without aeration. *Escherichia coli* strains DH5α (63) and MC1061 (16, 61) were used as an intermediate cloning host and were grown at 37°C on TY broth (27) with aeration (53). When appropriate, antibiotics were added to the media. For *L. plantarum*, 10 µg/ml chloramphenicol and 10 µg/ml or (for replica-plating) 30 µg/ml erythromycin was used. For *E. coli*, 10 µg/ml chloramphenicol and 250 µg/ml erythromycin were used.

Table 1. Strains and plasmids used in this study

Strains	Relevant features ^a	References
<i>L. plantarum</i>		
WCFS1	Single colony isolate of <i>L. plantarum</i> NCIMB8826	(28)
NZ5304	Derivative of WCFS1 containing a <i>lox66-P₃₂-cat-lox71</i> replacement of <i>bsh1</i> (<i>bsh1::lox66-P₃₂-cat-lox71</i>)	This work
NZ5305	Derivative of NZ5304 containing a <i>lox72</i> replacement of <i>bsh1</i> ($\Delta bsh1$)	This work
NZ5334	Derivative of WCFS1 containing a <i>lox66-P₃₂-cat-lox71</i> replacement of <i>melA</i> (<i>melA::lox66-P₃₂-cat-lox71</i>)	This work
NZ5335	Derivative of NZ5334 containing a <i>lox72</i> replacement of <i>melA</i> ($\Delta melA$)	This work
NZ5337	Derivative of NZ5335 containing a <i>lox72</i> replacement of <i>melA</i> and <i>bsh1</i> ($\Delta melA$, $\Delta bsh1$)	This work
NZ5338	Derivative of NZ5335 containing a <i>lox66-P₃₂-cat-lox71</i> replacement of part of <i>lacS2</i> ($\Delta melA$, <i>lacS2::lox66-P₃₂-cat-lox71</i>)	This work
NZ5339	Derivative of NZ5338 containing a <i>lox72</i> replacement of <i>melA</i> and part of <i>lacS2</i> ($\Delta melA$, $\Delta lacS2$)	This work
<i>E. coli</i>		
DH5α	Cloning host ; <i>F'</i> , $\Phi 80dlacZ\Delta M15$, <i>endA1</i> , <i>recA1</i> , <i>hsdR17</i> (r_k^- , m_k^+), <i>supE44</i> , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i> , $\Delta(lacZYA-argF)U169$, <i>deoR</i> , λ^-	(63)
MC1061	Cloning host ; <i>F'</i> <i>araD139</i> $\Delta(ara-leu)7696$ <i>galE15</i> <i>galK16</i> $\Delta(lac)X74$ <i>rpsL</i> (<i>Str^r</i>) <i>hsdR2</i> (r_k^- , m_k^+) <i>mcrA</i> <i>mcrB1</i>	(16, 61)
<i>L. lactis</i>		
MG1363	Plasmid-free derivative of NCDO 712	(19)

Table 1 continued

Plasmids	Relevant features ^a	References
pCR [®] -Blunt	Kan ^r ; cloning vector for blunt-end PCR products	Invitrogen
pGID023	Em ^r ; pJDC9 derivative containing the pE194 replication functions; unstable in lactobacilli	(24)
pGIZ850	Cm ^r , Em ^r , Ap ^r ; pUC18 derivative containing a P ₃₂ - <i>cat</i> cassette that is selectable at single copy level	(21)
pNZ273	Cm ^r ; pNZ124 carrying the promoterless <i>gusA</i> gene from <i>E. coli</i>	(47)
pNZ5315	Cm ^r , Em ^r ; pNZ7101 derivative containing a <i>lox66</i> site	This work
pNZ5317	Cm ^r , Em ^r ; pNZ5315 derivative containing a <i>lox71</i> site	This work
pNZ5318	Cm ^r , Em ^r ; pNZ5317 derivative for multiple gene replacements containing <i>las</i> and <i>pepN</i> terminators	This work
pNZ5319	Cm ^r , Em ^r ; pNZ5318 derivative for multiple gene replacements in Gram-positive bacteria	This work
pNZ5340	Cm ^r , Em ^r ; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 <i>mela</i>	This work
pNZ5325	Cm ^r , Em ^r ; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 <i>bshI</i>	This work
pNZ5344	Cm ^r , Em ^r ; pNZ5319 derivative containing homologous regions up- and downstream of <i>lacS2</i> in <i>mela::lox72</i> strain NZ5335	This work
pNZ5346	Cm ^r ; pNZ273 derivative containing the promoter region and ribosome binding site of lp_1144 of WCFS1	This work
pNZ5347	Cm ^r ; pNZ7110 derivative containing the promoter region and RBS of lp_1144, driving expression of <i>cre</i>	This work
pNZ5348	Em ^r ; pGID023 derivative containing <i>cre</i> under control of the lp_1144 promoter	This work
pNZ7101	Cm ^r , Em ^r ; pACYC184 derivative for gene replacements	(8)
pNZ7110	Ap ^r ; pUC18 derivative containing the <i>cre</i> gene	(9)
pNZ84	Cm ^r ; pACYC184 derivative	(59)

^a Kan^r, kanamycin resistant; Cm^r, chloramphenicol resistant ; Em^r, erythromycin resistant; Ap^r, ampicillin resistant.

Table 2. Primers used in this study

Primers	5' to 3' sequence
Bsh1fr1F	gattaagtttgcaggacatggag
Bsh1R	gccagccattggaacttactctg
Cat96F	tcaaatacagcttttagaactgg
Cat97R	accatcaaaaattgtataaagtggc
creF	ctaactcgagtgtacaccaattc
creR	ggctatcaatcaaagcaacacg
EryintF	cgataccggttacgaaattgg
EryintR	cttgctcataagtaacggtag
Lastermi_F	acgtccgcggggacaatatggggtaagcg
Lastermi_R	aagaagatctctaaagctgacggggtaaac
MellacF	accgttaagatgcgtgggactgg
MellacR	catagtaaaatcttcccttcgcta
pNZ84F	cgggatcccaacagtactgcgatgag
pNZ84R	gggggtaccatccagtattttttctcc
80	cgtttaacaatttaaatctaccgttcgtataatgtatgctatacgaagttagaca
81	ttgtcataacttcgtatagcatacattatacgaacggtagatttaattgtttaacgtgct
82	gaccataaacttcgtataatgtatgctatacgaacggtagacgcccgggcatgagctccgat
83	cggagctcatgcccgggctgtaccgttcgtatagcatacattatacgaagttagg
85	gttttttctagccaagctcaca
87	gccgactgtactttcggatcct
90	catagtaaaatcttcccttcgc
91	gtcgtaaaagtgttcttcttagc
92	gctaaggactaagctcagcc
93	gagtttaggactacagggggc
95	aatatgtgtacaggctgagcttagtccttagcc
101	gattgcgattgatatcgatggc
102	tatggcagtacacataactagtaatcctcc
103	tactatgcagttaactaaaagcc
104	cttaccatcatgcgtcccg
106a	gttcgataaagaatgaggatggc
107a	ttatcgcaagtatctcaaattgcg
108	tattggccttcccaccattagc
109	cacgttatttacggcgacggg
111	cgtgttgctttgattgatagcc
113	gctcgagcgcgttatcggtcctttaattgg
124	gcgcctcgagccgcaatcgctttgatttctgcc
125	tccgattgcattggcgagtcgg
126	taccgttaagatgcgtggg
127	ggcgggataccttatacgggtgacagcagacgg
128	cattaaaccaacggatggtcgcg
130	cgtggttgatggcatttggg
137	cttctaccattattacagca
1144F	ggatccgtcgaccgcgattttgtatgagatg
1144R	ggatccgctgttcgccaccctttcta

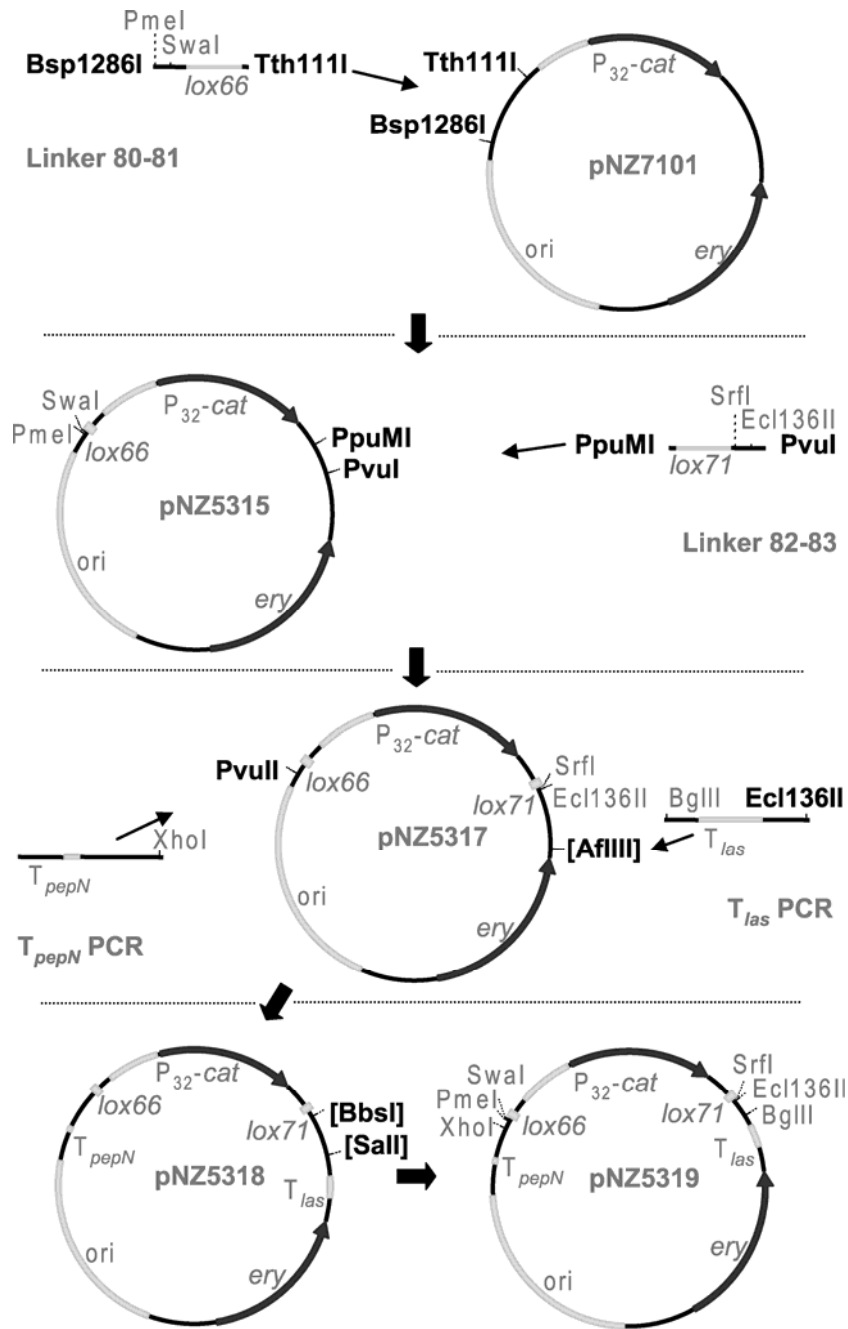
DNA manipulations. Plasmid DNA was isolated from *E. coli* on a small scale using the alkaline-lysis method (7). Large-scale plasmid DNA isolations were performed using Jetstar columns as recommended by the manufacturer (Genomed GmbH, Bad Oberhausen, Germany). For DNA manipulations in *E. coli*, standard procedures were used (53).

DNA isolation and transformation of *L. plantarum* was performed as described previously (25), with slight modifications. For DNA isolation, an overnight culture of *L. plantarum* WCFS1 was diluted 20 times in 50 ml of fresh MRS medium and cells were grown to an optical density at 600 nm (OD₆₀₀) of 1. Cells were pelleted by centrifugation for 10 minutes at 4500 rpm (Megafuge 1.0R, Heraeus, Hanau, Germany), resuspended in 2.5 ml of THMS buffer (30 mM Tris-HCl pH 8, 3 mM MgCl₂, 0.73 M sucrose) containing 50 mg/ml lysozyme and incubated for 2 hours at 37°C. Cells were pelleted by centrifugation and resuspended in 2.5 ml TE containing RNase. Subsequently, 125 µl of 10% SDS was added and cells were incubated for 15 minutes at 37°C. Then, 25 µl of 20 mg/ml proteinase K was added and the solution was subjected three times to phenol/chloroform extraction. The total DNA was precipitated with isopropanol, washed with 70% ethanol, dried and taken up in water. For transformation of *L. plantarum* WCFS1, a preculture in MRS broth was diluted in MRS broth containing 1% glycine and cells were grown to an OD₆₀₀ of 1. Cells were kept on ice for 10 minutes and pelleted by centrifugation for 10 minutes at 4000 rpm (Megafuge 1.0R, Heraeus, Hanau, Germany). Cells were then resuspended in ice cold 30% PEG-1450 and kept on ice for 10 minutes. Finally, cells were pelleted by centrifugation for 10 minutes at 4000 rpm and concentrated 100-fold into ice cold 30% PEG-1450. Subsequently, 40 µl of cell suspension and up to 5 µl of plasmid DNA solution was electroporated using a GenePulser Xcell electroporator (Biorad, Veenendaal, The Netherlands) in cuvettes with a 2 mm electroporation gap at 1,5 kV, 25 µF capacitance and 400 Ω parallel resistance. Restriction endonucleases, *Taq*, *Pfx* and *Pwo* DNA polymerases, T4 DNA ligase, and Klenow enzyme were used as specified by the manufacturers (Promega, Leiden, The Netherlands, and Boehringer, Mannheim, Germany). Primers were obtained from Genset Oligos (Paris, France).

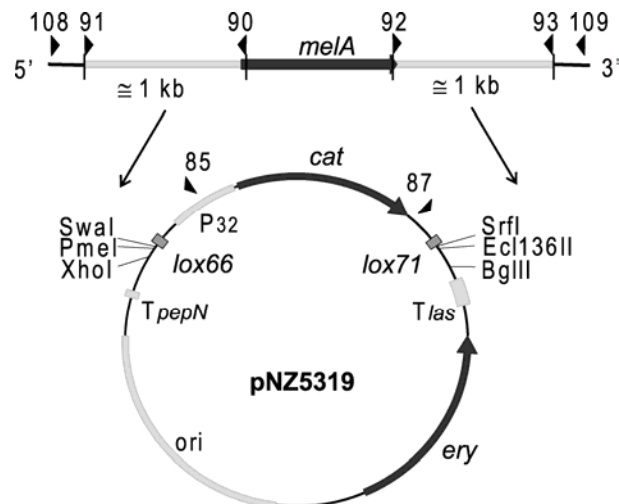
Mutagenesis vector construction. To facilitate construction of chromosomal gene replacements, the universal mutagenesis vector pNZ5319 was constructed (Figure 2A).

A Cre-lox-based system for multiple gene deletions and selectable-marker removal

A



B



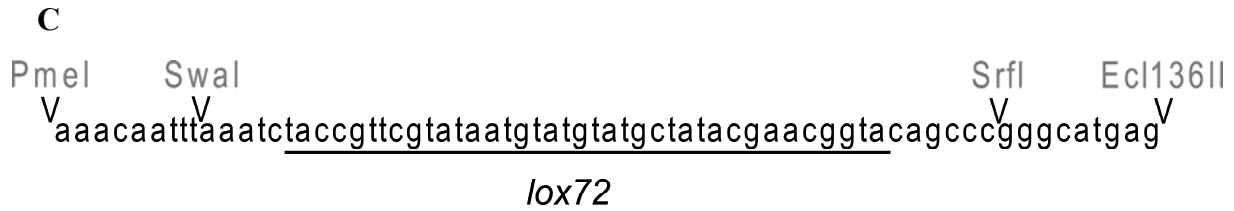


Figure 2. Panel A. Schematic representation of the construction of mutagenesis vector pNZ5319. 80 and 81 linkers were annealed (Table 2) and cloned into pNZ7101 digested with Bsp1286I and Tth111I, yielding pNZ5315 (Table 1). Subsequently, 82 and 83 linkers (Table 2) were annealed and cloned into pNZ5315 digested with PpuMI and PvuI, yielding pNZ5317 (Table 1). Subsequently, the *las* terminator (38, 39) and *pepN* terminator (58) were amplified by PCR from *L. lactis* MG1363 (19) using primers LasTermi_F and LasTermi_R, 111 and 113, respectively (Table 2). The PCR fragment containing the *las* terminator and BglII restriction site was digested with Ecl136II and cloned in pNZ5317 digested with AflIII and treated with Klenow enzyme. Subsequently, the PCR fragment containing the *pepN* terminator and XhoI restriction site was cloned into the PvuII site of the targeting vector, yielding pNZ5318 (Table 1). Finally, non-functional DNA sequences were removed by BbsI and SalI digestion, treatment with Klenow enzyme and self-ligation, resulting in pNZ5319 (Table 1). Panel B. Schematic representation of mutagenesis vector pNZ5319. Indicated are the pACYC184-derived origin of replication (*ori*), the erythromycin (*ery*) resistance gene, the chloramphenicol resistance gene under control of the P_{32} promoter (P_{32} -*cat*), flanked by *lox66* and *lox71* sites, and the lactococcal T_{las} and T_{pepN} terminators. The presence of rare-cutting blunt-end restriction sites SwaI, PmeI, SrfI and Ecl136II allow direct cloning of blunt-end PCR products of the flanking regions of the target locus. As an example, the regions used for PCR amplification and cloning into pNZ5319 for construction of a *L. plantarum melA* mutant are indicated. In addition, the sticky-end restriction sites XhoI and BglII can be used in combination with the blunt-end restriction sites. The presence of two selectable marker gene cassettes (P_{32} -*cat* and *ery*) on the mutagenesis vector allows direct selection of double cross-over integrants based on their antibiotics resistance (Cm^r) and sensitivity (Em^s) phenotype. Primers used in this study are indicated as black arrowheads. Panel C. Schematic representation of the in-frame insertion that is left in the genome after *lox72*-replacement of the target gene. Depending on the restriction sites used for cloning of the homologous DNA fragments (as indicated in the figure) that encompass a whole number of codons of the target gene, the number of foreign nucleotides left in the genome is 45 (cloning using SwaI and SrfI restriction sites), 54 (PmeI/SrfI or SwaI/Ecl136II), or 63 (PmeI/Ecl136II), thereby creating an in-frame deletion of the target gene.

For this, the pACYC184-derived origin of replication was amplified by PCR (using *Pfx* polymerase, pNZ84F and pNZ84R primers and pNZ84 (59) as template DNA) and cloned into the NaeI restriction site of pGIZ850 (21), resulting in pNZ7101 (8). To introduce *lox66* and blunt-end restriction sites SwaI and PmeI, 80 and 81 linkers (Table 2), which were annealed and cloned into the Bsp1286I and Tth111I restriction sites upstream of the P_{32} -*cat* cassette of pNZ7101 (8), yielding pNZ5315 (Table 1). Subsequently, 82 and 83 linkers (Table 2), which contained *lox71* and blunt-end restriction sites Ecl136II and SrfI, were annealed and cloned into the PpuMI and PvuI restriction sites downstream of the P_{32} -*cat* cassette of pNZ5315, yielding pNZ5317 (Table 1). Furthermore, both the *las*-operon and *pepN* terminator regions (38, 39) were amplified by PCR from *L. lactis* MG1363 (19) using primers LasTermi_F and LasTermi_R, 111 and 113, respectively (Table 2). The PCR fragment containing

the *las* terminator and BglII restriction site was digested with Ecl136II (to avoid introduction of an extra Ecl136II restriction site into the mutagenesis vector) and cloned in pNZ5317 that was digested with AflIII and treated with Klenow enzyme to generate blunt ends. Subsequently, the PCR fragment containing the *pepN* terminator and XhoI restriction site was cloned into the PvuII restriction site of the targeting vector, yielding pNZ5318 (Table 1). Removal of residual and non-functional DNA sequences from the pNZ5318 mutagenesis vector was achieved by BbsI and SalI digestion, treatment with Klenow enzyme to generate blunt ends, and self-ligation, yielding pNZ5319 (Table 1).

Construction of gene-specific mutagenesis vectors. For construction of gene-specific mutagenesis vectors, a standard cloning procedure was used (Figure 2B). Typically, a fragment of 1kb of the up- and of the downstream sequence of the target locus was amplified by PCR using a proofreading polymerase and cloned in blunt-end restriction sites SwaI or PmeI and Ecl136II or SrfI of pNZ5319, respectively. When desired, sticky-end restriction sites XhoI or BglII can be used in combination with the blunt-end restriction sites. To ultimately obtain in-frame gene deletions (following Cre recombination, see below and Figure 2C), primers were designed in such a way that the 5'- and 3'-flanking regions of the target gene encompassed the first and last five codons of that gene, respectively. Cloning efficiency of the PCR amplification products of the 5'- and 3'-flanking regions of the target gene into the mutagenesis vector was enhanced by removal of self-ligation vector in the ligation mixture by digestion with SwaI, PmeI, Ecl136II, or SrfI, depending on the restriction site used for cloning. Colonies harboring the anticipated insert in the desired orientation could be identified effectively by colony PCR using a vector-specific primer (annealing to the P_{32} -*cat* region: for cloning of 5' sequences in SwaI or PmeI, reverse primer 85; for cloning of 3' sequences in Ecl136II or SrfI, forward primer 87), combined with an insert-specific primer (see also below) (Table 2). Following the strategy described above, the *mela* mutagenesis vector pNZ5340 was constructed by successive cloning of the 5' and 3'-flanking regions of *mela* (lp_3485) (28) (Figure 2B) into the SwaI and Ecl136II restriction sites of pNZ5319, (amplified by PCR using *Pfx*, *L. plantarum* WCFS1 genomic DNA as a template, and primer sets 91 and 90, 92 and 93, respectively (Table 2)). Clones that harbored the anticipated inserts were identified by PCR using primer sets 91 and 85, 87 and 93, respectively (Table 2 and Figure 2B).

Likewise, the *bshI* replacement vector pNZ5325 was constructed by the successive cloning of PCR products of the 5' and 3'-flanking regions of WCFS1 *bshI* (lp_3536) (28) (amplified using *Pfx* polymerase, WCFS1 genomic DNA as a template, and primer sets 101 and 102, 103 and 104, respectively (Table 2)) into pNZ5319 digested with SwaI and Ecl136II, respectively. Clones that harbored the correct inserts were identified by PCR using primer sets 101 and 85, 87 and 104, respectively (Table 2).

The third locus *lacS2* (lp_3486) (28) was targeted for mutagenesis in a *L. plantarum* WCFS1 $\Delta melA$ background (NZ5335; Table 1). For construction of the corresponding mutagenesis vector pNZ5344, PCR products of 5' and 3' regions of *lacS2* (amplified using *Pfx* polymerase, $\Delta melA$ (NZ5335) template DNA, and primer sets 124 and 125, 126 and 127, respectively (Table 2)) were digested with XhoI and BamHI, respectively and sequentially cloned in XhoI, SmaI and BglII, Eco136II digested pNZ5319. Clones harboring the correct insert were identified using primer sets 124 and 85, 87 and 127, respectively (Table 2).

Mutant construction. In order to engineer *lox66-P₃₂-cat-lox71* gene replacements, 4 μ g of the appropriate mutagenesis vector was transformed into *L. plantarum* WCFS1 by electroporation, as described previously (25). Chloramphenicol-resistant (Cm^r) transformants were selected and replica-plated to check for an erythromycin-sensitive (Em^s) phenotype. Candidate double cross-over clones (Cm^r, Em^s) were analyzed by PCR amplification of the *cat* and *ery* gene, using cat96F/cat97R and eryintF/eryintR primers, respectively (Table 2). Correct integration of the *lox66-P₃₂-cat-lox71* cassette into the genome (for *melA* replacement, see Figure 3A) was confirmed by PCR amplification of the flanking regions of the integrated *lox66-P₃₂-cat-lox71* cassette, using primers annealing uniquely to genomic sequences (for *melA*, primer 108 for amplification of the 5'-region and primer 109 for amplification of the 3'-region) combined with the mutagenesis vector-specific primers 85 and 87 that annealed to the P₃₂-*cat* region, respectively (Table 2 and Figure 3A). Likewise, for analysis of the flanking regions of the *lox66-P₃₂-cat-lox71* replacement of *bshI*, primers 106a and 85, 87 and 107a were used; for *lacS2* replacement, primers 130 and 85, 87 and 109 were used (Table 2 and Figure 3A)).

Transient Cre expression vector. For expression of Cre, a 100 bp region upstream of the *L. plantarum* WCFS1 gene lp_1144 (28), containing the functional promoter P₁₁₄₄ (11), was amplified by proofreading PCR using 1144F and 1144R primers and cloned into pCR[®]-Blunt (Invitrogen, Breda, the Netherlands) (Table 1 and 2). To assess the promoter activity of the P₁₁₄₄ fragment, it was digested from the pCR[®]-Blunt vector with BamHI and cloned upstream of the *gusA* gene into BglII digested pNZ273 (47), resulting in pNZ5346. Quantitative β -glucuronidase activity measurements were performed as described previously (17). To obtain *cre* expression, the promoter-fragment was digested from pNZ5346 with SalI and BamHI and cloned upstream of *cre* into SalI, BamHI digested pNZ7110 (9), yielding pNZ5347. Finally, the P₁₁₄₄ promoter-*cre* cassette was digested from pNZ5347 using KpnI and HindIII and cloned into correspondingly digested pGID023, yielding pNZ5348, which is unstable in lactobacilli (24).

The stability of the pGID023 replicon in *L. plantarum* was determined in duplicate by culturing for 10 generations without selection pressure. Subsequently, cells were plated with and without selection pressure and the amount of colony forming units (CFU)/ml was determined. The presence of

pGID023 in *L. plantarum* was verified by colony PCR, using eryintF and eryintR primers (Table 2).

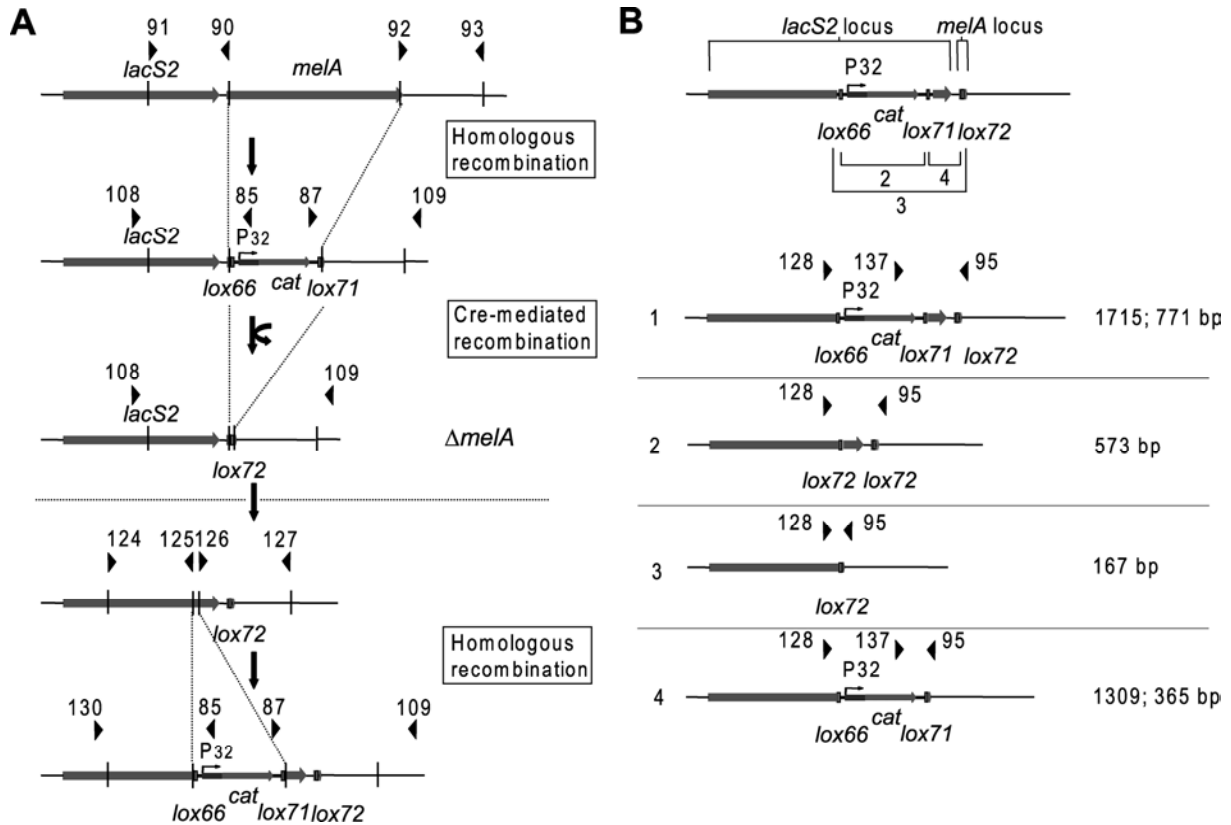


Figure 3. Panel A. Strategy for construction of double cross-over mutants and subsequent Cre-lox-mediated selectable-marker removal (*melA* and subsequent *lacS2* mutagenesis are shown as an example). Subsequent to transformation of the gene-specific mutagenesis vector, the target gene (*melA*) is replaced by a *lox66-P₃₂-cat-lox71* cassette by homologous recombination. After selection of the double cross-over mutant, the *lox66-P₃₂-cat-lox71* cassette is resolved to a single double-mutant *lox72* site by transient Cre expression from a curable plasmid. Subsequently, the next round of gene replacement (*lacS2*) can be performed. Primers that were used for amplification of the homologous DNA fragments used for targeting cloning in the mutagenesis vector pNZ5319 (91 and 90; 92 and 93 for *melA* targeting; 124 and 125; 126 and 127 for *lacS2* targeting), confirmation of correct integration of the mutagenesis vector into the genome (108 and 85; 87 and 109 for *melA* targeting; 130 and 85; 87 and 109 for *lacS2* targeting) and confirmation of Cre resolution of the *lox66-P₃₂-cat-lox71* cassette (108 and 109 for *melA* targeting) are indicated by black arrowheads. Panel B. Possible products of Cre recombination during multiple gene replacement in a single genetic background, as exemplified by recombination of the *lox66-P₃₂-cat-lox72* at the *lacS2* locus in a *ΔmelA* background. Primers that were used for distinction of the four possible products of *lacS2::lox66-P₃₂-cat-lox72* recombination in a *ΔmelA* background by PCR are indicated by black arrowheads (128, 137 and 95 in one reaction mixture). The corresponding PCR product sizes are indicated on the right. (1). No recombination occurred; (2). Correct recombination occurred, removing the *P₃₂-cat* selectable marker cassette. (3). Incorrect recombination between *lox66* and *lox72* occurred, resulting in deletion of the intermediate region. (4). Incorrect recombination between *lox71* and *lox72* occurred, resulting in deletion of the intermediate region.

Cre-mediated mutant locus resolution. To excise the P₃₂-*cat* selectable marker cassette from the chromosome, 4 µg of the transient erythromycin-selectable *cre* expression plasmid pNZ5348 was transformed into *lox66*-P₃₂-*cat*-*lox71* gene replacement mutants. After 48 to 72 hours of growth, Em^r colonies were checked by PCR for the presence of cells that had undergone Cre-mediated recombination, using primers spanning the recombination locus (specifically, using the following primers: for *mela*, 108 and 109 (Figure 3A); for *bsh1*, *bsh1fr1F* and *bsh1R*; for *lacS2*, 128, 137 and 95 in one PCR reaction (Figure 3B);(Table 2)). The pNZ5348 vector was cured from appropriate colonies of *L. plantarum* mutants by growing without erythromycin selection pressure for 10 generations. To obtain clonal strains, single colony isolates were selected in which curing of the Cre expression vector was confirmed by absence of PCR amplification of *ery* (using primers *eryintF* and *eryintR*) and *cre* (using primers *creF* and *creR*) (Table 2) and Cre-mediated recombination was confirmed by PCR amplification as stated above. Additionally, the presence of a correctly resolved *lox72* (Figure 2C) site in these PCR products was confirmed by sequencing (Baseclear, Leiden, the Netherlands) using primers 95, *bsh1fr1F*, and 95 for *mela*, *bsh1*, and *lacS2* replacement, respectively.

Southern blot analysis. To confirm the genotype of WCFS1 mutant derivatives *mela::lox66*-P₃₂-*cat*-*lox71* (NZ5334), Δ *mela* (NZ5335), Δ *mela*, *lacS2::lox66*-P₃₂-*cat*-*lox71* (NZ5338), and Δ *mela* Δ *lacS2* (NZ5339) (Table 1), Southern blot analysis was performed as described earlier (53) using *Ava*I and *Dra*I digests of total DNA. As a probe, a PCR amplification product of the intergenic region of *mela* and *lacS2* (amplified with *Taq* polymerase, WCFS1 total DNA and primers *mellacF* and *mellacR*, respectively) was used.

HPLC assay of bile salt hydrolase activity. To determine bile salt hydrolase activity of *L. plantarum*, an overnight culture was inoculated 1:10 in fresh MRS medium and cells were grown to an OD₆₀₀ of 5. Cells were pelleted by centrifugation for 10 minutes at 4500 rpm (Megafuge 1.0R, Heraeus, Hanau, Germany) and resuspended in MRS medium to an OD₆₀₀ of 100. For determination of bile salt hydrolase activity, wild type *L. plantarum* cells were diluted in MRS to an OD₆₀₀ of 10, whereas cells of *bsh1* deletion strains were used undiluted. Conversion of the bile salt glycocholic acid (Sigma, Zwijndrecht, the Netherlands) was determined by HPLC as described previously (18). Separations were carried out with a reversed-phase resin-based column (PLRP-S, 5 µm, 300 Å, 250 x 4.6 mm I. D., Polymer laboratories, Shropshire, UK) and matching pre-column. Bile salts were detected using a pulsed amperometric detector (EG&G Princeton applied research, Princeton, NJ), equipped with a gold working electrode and a reference electrode (Ag/AgCl).

Nucleotide sequence accession number. The sequence of the mutagenesis vector pNZ5319 is available in the GenBank database under accession number DQ104847.

The sequence of the P₁₁₄₄-*cre* cassette of the *cre* expression plasmid pNZ5348 is available in the GenBank database under accession number DQ340306.

RESULTS

Strategy of gene replacement and selectable-marker removal. For generation of gene deletions, the mutagenesis vector pNZ5319 (Figure 2B) was constructed and implemented in the Gram-positive model organism *Lactobacillus plantarum* WCFS1 (28). This medium copy *E. coli* cloning vector contains a PACYC184 origin of replication, which is suitable for 'suicide' mutagenesis in lactic acid bacteria, as described earlier (17, 59).

Cloning of PCR-amplified homologous DNA fragments up- and downstream of the mutagenesis locus (necessary for targeting of the mutagenesis vector to the genomic locus) in pNZ5319 is facilitated by the presence of rare-cutting blunt-end restriction sites PmeI, SmaI, SrfI and Ecl136II in the vector, flanked by the lactococcal *las* (38, 39) and *pepN* (58) terminators (Figure 2B).

Furthermore, the mutagenesis vector pNZ5319 contains both a chloramphenicol (*lox66*-P₃₂-*cat*-*lox71*) and erythromycin (*ery*) resistance cassette that can be selected at single copy chromosomal level. Following transformation of the mutagenesis vector to the target organism, the antibiotic resistance cassettes allow for direct selection of double cross-over mutants on basis of their antibiotic resistance and sensitivity phenotype. In the resulting double cross-over mutant strains, the target gene is replaced by a *lox66*-P₃₂-*cat*-*lox71* cassette. The presence of the *lox* sites renders the P₃₂-*cat* cassette excisable from the genome of the double-cross over mutant strain by Cre recombinase. Using native *loxP* sites, multiple gene deletions would lead to the integration of multiple *loxP* sites into the genome that can cause genomic instability in the presence of Cre (14, 15, 57). Therefore, *loxP* sites containing mutations within the inverted repeats (*lox66* and *lox71*) (4) were used (Figure 1) that after Cre recombination result in a double-mutant *loxP* site (*lox72*), which shows strongly lowered affinity for Cre.

Transient Cre expression was driven by P₁₁₄₄ (upstream of gene *lp_1144*) from the vector pNZ5348, which contains a replicon that is unstable in lactobacilli (24). Although this plasmid can be introduced in *L. plantarum* when selective (Em^r) conditions are maintained, its intrinsic instability in this host resulted in rapid curing of the plasmid when selective pressure was relieved. In our experiments, an approximate 1000-fold reduction in plasmid retention was obtained by culturing for 10 generations in absence of selection pressure, as determined by antibiotic resistance profiling and PCR (data not shown). Finally, the P₁₁₄₄ promoter is predicted to drive constitutive, moderate levels of transcription of the downstream gene (*pcrA*), encoding a DNA helicase. Previous experiments in our laboratory using *gusA* (which encodes β-

glucuronidase) as a promoter probe confirmed the prediction of the characteristics of the P₁₁₄₄ promoter (data not shown).

Notably, in our design, the PCR-amplified homologous DNA fragments used for locus targeting include a whole number of codons of the 5' and 3' ends of the gene targeted for mutagenesis. Thus, depending on the restriction sites in pNZ5319 that were used for cloning of the 5' and 3' homologous regions, the total number of foreign nucleotides left in the genome after Cre-mediated excision of the selectable marker cassette is 45 (using SrfI and SwaI), 54 (using SrfI and PmeI or Ecl136II and SwaI) or 63 (using Ecl136II and PmeI), thereby generating an in frame deletion (Figure 2C).

Single locus mutagenesis. To validate the gene deletion system, the *mela* gene (lp_3485) and *bsh1* gene (lp_3536) of *L. plantarum* WCFS1 (28) were chosen as target genes for single locus mutagenesis. The *mela* gene encodes an alpha-galactosidase (EC3.2.1.22), which is predicted to be involved in hydrolysis of the sugar melibiose into galactose and glucose. In *L. plantarum*, the *mela* gene is induced by melibiose and repressed by glucose (56). The *mela* gene is a convenient target for mutagenesis, since it is predicted to encode a non-redundant function in *L. plantarum* WCFS1 and its phenotype is likely to be measurable both quantitatively (hydrolysis of a chromogenic substrate) and qualitatively (absence of growth on melibiose as a sole carbon source). The *bsh1* gene of *L. plantarum* WCFS1 is predicted to encode a bile salt hydrolase (Bsh; EC3.5.1.24). Bile salt hydrolases catalyze cleavage of the amino acid moiety from the steroid nucleus of conjugated bile salts. The DNA sequence of *bsh1* of *L. plantarum* WCFS1 is 99% identical to the sequence of the *bsh* gene of *L. plantarum* LP80, of which a previously constructed *bsh* mutant derivative was shown to be deficient in bile salt hydrolase activity (35). However, the *L. plantarum* WCFS1 genome appears to be four-fold redundant for this function, containing genes annotated as *bsh1* to *bsh4* (28). The *L. plantarum* *bsh1* gene was selected as a target for mutagenesis to investigate whether it is the sole bile salt hydrolase-encoding gene in this strain.

For construction of *mela* and *bsh1* mutant strains, gene-specific mutagenesis vectors pNZ5340 and pNZ5325 were constructed by direct cloning of PCR-amplified homologous DNA fragments up- and downstream of *mela* or *bsh1*, respectively, and transformed to *L. plantarum* WCFS1. The genetic events involved are schematically illustrated for the *mela* locus in Figure 3A. During primary selection of *mela* double cross-over mutants, a number of 34 Cm^r colonies was found, of which 9 appeared to display the Em^s phenotype correlated to the *mela::lox66-P₃₂-cat-lox71* genotype. A single colony isolate was selected and designated NZ5334 (Table 1). For *bsh1* mutagenesis, a slightly lower number of 12 Cm^r colonies was found during primary selection of double cross-over mutants, of which the relative amount of colonies displaying an Em^s phenotype (3; correlating to a *bsh1::lox66-P₃₂-cat-lox71* genotype) appeared to be similar to that observed for the *mela* locus. A single colony isolate was

selected and designated NZ5304 (Table 1). Correct integration of the *lox66-P₃₂-cat-lox71* cassette into the genome was confirmed by PCR and by Southern blot analysis (Figure 4).

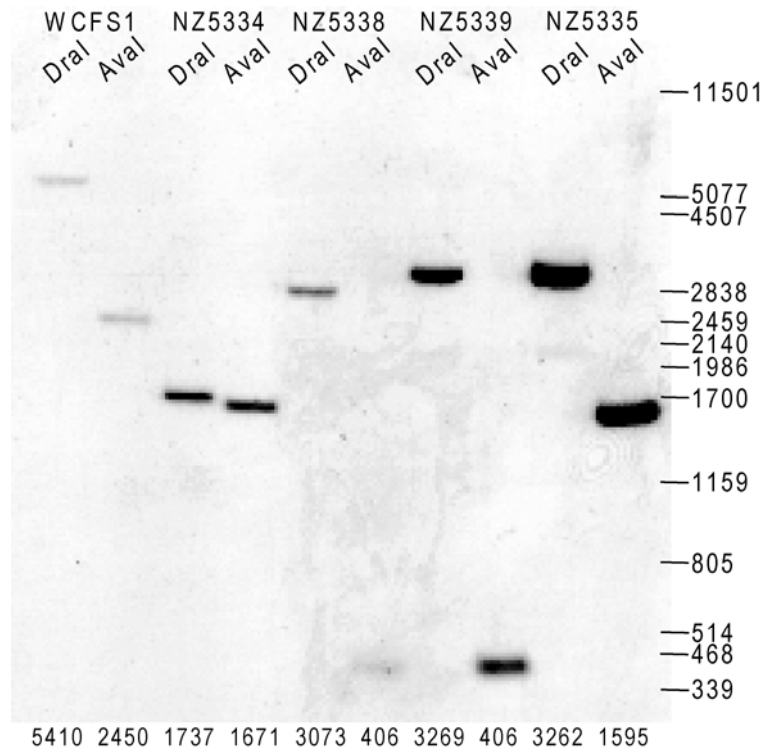


Figure 4. Southern blot analysis of WCFS1 and *melA::lox66-P₃₂-cat-lox71* (NZ5334), Δ *melA*, *lacS2::lox66-P₃₂-cat-lox71* (NZ5338), Δ *melA*, Δ *lacS2* (NZ5339), and Δ *melA* (NZ5335) derivatives. The restriction enzymes (DraI and AvaI, respectively) used for restriction of genomic DNA of the strains were chosen in such a way that all strains could be distinguished based on the predicted size of the hybridizing bands (indicated at the bottom of the figure in bp). The size of the hybridizing bands of the various strains was as expected, as can be determined by the size of the bands of the DNA ladder (indicated at the right of the figure in bp). DraI; DraI digestion of total DNA; AvaI; AvaI digestion of total DNA.

To resolve the *lox66-P₃₂-cat-lox71* cassette at the mutation locus to a single in-frame *lox72* site, the erythromycin-selectable Cre expression plasmid pNZ5348 was transformed to the NZ5334 and NZ5304 double cross-over mutant strains. After 48 hours of incubation, a number of 22 Em^s colonies was analysed, of which 9 (for the *melA*-mutagenised stain) and 15 (for the *bsh1*-mutagenised strain) colonies were found to contain cells that had undergone Cre recombination, as determined by PCR. Subsequently, the Cre expression vector was allowed to be lost from the cells by culturing without selective pressure. Single colony isolates (designated NZ5335 (Δ *melA*), and NZ5305 (Δ *bsh1*) (Table 1)) were selected and analysed for loss of the Cre expression vector and correct Cre-mediated recombination of the target loci, as determined by Em^s

phenotype, PCR (Figure 3A), DNA sequencing of the mutated locus and Southern blot analysis (Figure 4).

To further confirm the genotypes of these mutants, the anticipated physiologic consequences were analysed. As expected, growth on MRS plates of the *L. plantarum* WCFS1 *mela* deletion mutant NZ5335 in the presence of glucose as a carbon and energy source was unaffected as compared to the wild type. However, in the presence of melibiose as a sole carbon and energy source, growth on MRS plates of the *L. plantarum* WCFS1 *mela* deletion mutant NZ5335 was completely abolished, whereas growth of the wild type strain was normal (data not shown). This confirms the lack of functional redundancy for this gene in the *L. plantarum* WCFS1 genome and indicates that *mela* is essential for growth on melibiose. In analogy, quantitative determination of bile salt hydrolase activity of the *bshI*-deletion mutant NZ5305 revealed that *bshI*-deficiency leads to a 90 % reduction of bile salt hydrolase activity in *L. plantarum* WCFS1 (Figure 5).

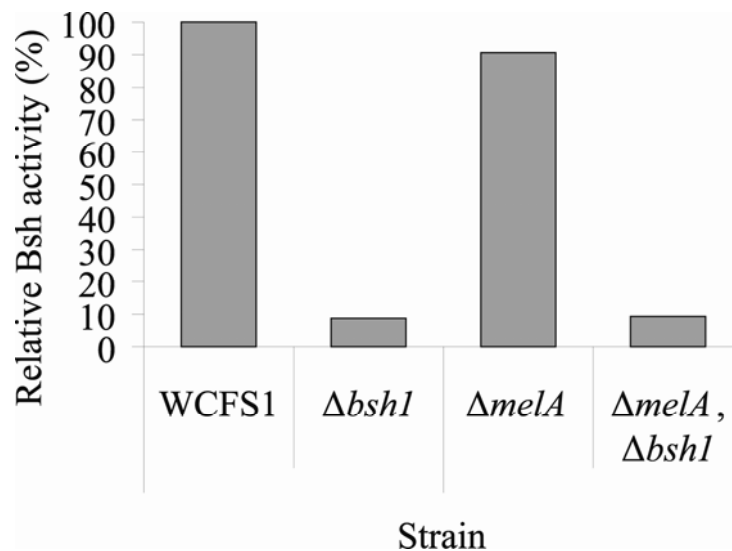


Figure 5. Bsh activity of $\Delta bshI$ (NZ5303), $\Delta mela$ (NZ5335), and $\Delta mela, \Delta bshI$ (NZ5337) strains relative to WCFS1. Deletion of *bshI* in WCFS1 or NZ5335 results in a 90 % reduction of Bsh activity.

Mutagenesis of genetically unlinked loci in a single genetic background. To establish that the mutagenesis system constructed was indeed suitable for efficient construction of successive selectable marker-free gene deletions, the *bshI* gene was mutated in the $\Delta mela$ derivative (NZ5335) of *L. plantarum* WCFS1.

After transformation of the *bshI* mutagenesis vector pNZ5325 into NZ5335 (Table 1), a number of 38 Cm^r colonies was found during primary selection of double cross-over integrants, of which 4 colonies displayed an Em^s phenotype correlating to a *bshI::lox66-P₃₂-cat-lox71* genotype (as confirmed by PCR). Subsequently, the *lox66-P₃₂-cat-lox71* cassette in the $\Delta mela, bshI::lox66-P_{32}$ -

cat-lox71 strain was efficiently resolved to a single in-frame *lox72* site by transient Cre expression from the erythromycin-selectable vector pNZ5348. After 72 hours of incubation, all of 15 Em^r colonies that were analysed contained cells that had undergone Cre-mediated recombination as confirmed by PCR. Following removal of the Cre expression vector by culturing without selection pressure, single colony isolates were taken and designated NZ5337 (*L. plantarum* $\Delta melA$, $\Delta bshI$). Loss of the Cre expression vector and correct recombination of the target locus was confirmed by PCR and DNA sequencing of the mutated locus. Furthermore, the anticipated double mutant ($\Delta melA$, $\Delta bshI$) phenotype could be confirmed, i.e. lack of growth on melibiose as a sole carbon and energy source and strongly reduced Bsh activity (Figure 5).

Mutagenesis of genetically linked loci in a single genetic background.

The approaches described above showed that mutagenesis of two genetically unlinked loci (*melA* and *bshI*) could be performed effectively. However, secondary gene replacement at a locus that is closely linked to the initially mutated locus leads to close proximity of the *lox66*-P₃₂-*cat-lox71* cassette and the *lox72* site of the primarily targeted locus (Figure 3), which could lead to incorrect resolution by the Cre enzyme. Provided that Cre recognizes all available *lox* sites with a certain affinity, four different modes of resolution of the locus could occur that involve *lox66* and *lox71* sites alone, or that involve *lox66* and/or *lox71* sites in combination with the *lox72* site. These modes of resolution can be distinguished by PCR analysis of resulting individual clones (Figure 3B). To evaluate the frequency of these potential artifact resolutions, a small part of the WCFS1 *lacS2* gene (lp_3486) (28), which is located directly upstream of *melA* (lp_3485) and is orientated in the same orientation as *melA*, was chosen as a secondary target for mutagenesis in the WCFS1 $\Delta melA$ strain NZ5335. In presence of glucose as a carbon and energy source, mutation of *lacS2* was not expected to affect growth. The *lacS2*-specific mutagenesis vector pNZ5344 was constructed by cloning of PCR-amplified homologous DNA fragments into the mutagenesis vector pNZ5319. Following transformation of pNZ5344 into the $\Delta melA$ strain, double cross-over integrants were selected based on their Cm^r and Em^s phenotype. Out of 22 Cm^r transformants, 12 clones displayed an Em^s phenotype and the corresponding $\Delta melA$, *lacS2::lox66-P₃₂-cat-lox71* genotype, as could be confirmed by PCR and Southern blot analysis (Figure 4). After Cre mediated resolution, recombination patterns could be distinguished by PCR using primers 128, 137 and 95 in a single PCR reaction (Table 2; Figure 3B). Of the 192 colonies that were analysed, 179 gave a PCR product, of which the vast majority (126 colonies; 70.4 %) appeared to have undergone correct Cre recombination ($\Delta melA$, $\Delta lacS2$), while almost all of the residual colonies (52 colonies; 29.1 %) appeared to contain a mixed population of correctly resolved ($\Delta melA$, $\Delta lacS2$) and unresolved cells ($\Delta melA$, *lacS2::lox66-P₃₂-cat-lox71*). Only a single colony (0.6 %) was detected that had

undergone incorrect recombination between the *lox66* site at the *lacS2* locus and the *lox72* site at the *melA* locus, while no colonies were detected that had undergone incorrect recombination between the *lox71* site at the *lacS2* locus and the *lox72* site at the *melA* locus. To further establish correct Cre-mediated resolution, the genotype of a single $\Delta melA$, $\Delta lacS2$ colony (designated NZ5339) was confirmed by DNA sequencing of the mutated locus and Southern blot analysis (Figure 4). Taken together these experiments support the robustness and selectivity of Cre-mediated resolution of the mutant *loxP* sites introduced in the genome, and exemplify the suitability of the mutagenesis system presented here for the construction of multi-locus mutants in a single genetic background.

DISCUSSION

Here we describe the construction of an effective Cre-*lox*-based toolbox for multiple gene replacements in a single genetic background. The procedure consists of three steps. In the first step, a gene-specific mutagenesis vector is constructed using standard cloning procedures. In the second step, the target gene is replaced by a *lox66*-P₃₂-*cat*-*lox71* cassette by double cross-over recombination. In the third step, the *lox66*-P₃₂-*cat*-*lox71* cassette introduced at the target locus is resolved by transient Cre expression, resulting in an in-frame *lox72* site in the genome (Figure 1 and 2C).

The mutagenesis vector presented facilitates efficient direct cloning of blunt-end PCR amplification products that represent homologous 5'- and 3'-flanking regions of any desired target locus in rare-cutting blunt-end restriction sites in the medium-copy *E. coli* cloning vector. The presence of two (single copy) selectable marker gene cassettes on the mutagenesis vector enables direct selection of double cross-over integrants. Direct selection of mutants provides a major advantage in procedures aiming to generate gene mutations that might result in growth retardation, where mutants may not be obtained when a method that implements a single selectable marker on the mutagenesis vector (i.e., pUC18 or pG⁺host) is used (12, 64).

However, the presence of a selectable marker in the chromosome hampers multiple gene replacements and may influence the expression of surrounding genes. As opposed to most mutagenesis systems that enable direct selection of double cross-over mutants by implementation of two selectable markers on the mutagenesis vector (such as pUC18ery) (60), the method described here adds the option of selectable-marker removal from the genome by transient Cre expression in the gene replacement background using an unstable and easily curable Cre-expressing vector that ensures removal of Cre activity before introduction of additional mutations. Thus, our system provides an important advantage over mutagenesis systems for Gram-positive bacteria that have been described to date.

Targeting of the *mela* and *bshI* locus of the Gram-positive model organism *L. plantarum* WCFS1 (28) showed the effectiveness of our system for construction of single locus double cross-over mutants and subsequent marker removal. The *mela* gene was shown to be essential for growth of *L. plantarum* WCFS1 on melibiose as a sole carbon and energy source. Although predicted to be four-fold redundant, the *bshI* gene appeared to be the major bile salt hydrolase-encoding gene in *L. plantarum* WCFS1. This is in good agreement with the finding that *bshI* of *L. plantarum* WCFS1 is almost identical to the *bsh* gene of *L. plantarum* LP80, of which a previously constructed mutant was shown to be bile-salt hydrolase deficient (35). However, to determine whether the *bsh2*, *bsh3*, and/or *bsh4* play a role in the remaining Bsh activity in the $\Delta bshI$ derivative of strain WCFS1, further investigation is required.

Furthermore, an essential characteristic for the successful generation of multiple gene deletions by Cre-lox recombination depends on correct resolution events even in presence of previously integrated *lox72* sites. In the work presented here, the efficiency of Cre-lox-based removal of the P₃₂-*cat* selectable marker cassette in multiple gene replacements was determined by successive mutagenesis of genetically unlinked and closely linked chromosomal loci. Thus, *bshI* was effectively deleted in an *L. plantarum* WCFS1 $\Delta mela$ strain, resulting in a $\Delta mela$, $\Delta bshI$ double mutant strain, which displayed the anticipated combination of the single-locus mutant phenotypes. During successive mutagenesis of the genetically closely linked loci *mela* and *lacS2* in *L. plantarum* WCFS1 (Figure 3), Cre-mediated resolution of *lox66* and *lox71* in close proximity of a *lox72* site was shown to occur correctly in 99.4 % of the colonies that were successfully analysed. Moreover, no colonies were found to have undergone incorrect resolution of *lox71* and *lox72*. However, the latter type of recombination (*lox71* and *lox72*) results in a *lox71* site, which in its turn can recombine with the still remaining *lox66* site, thereby generating a single *lox72* site (Figure 3B). This situation is indistinguishable from direct recombination of *lox66* and *lox72*, which was found to occur in only a single colony tested (0.6 %). The fact that correct Cre resolution of *lox66* and *lox71* occurs almost exclusively even in the close proximity of a *lox72* site emphasizes the selectivity of the Cre enzyme and underlines the advantage of this system relative to mutagenesis systems that employ native *loxP* sites, as have been used in some Gram-negative bacteria (41, 46, 49). Especially in prokaryotes, where only one chromosome is present, the use of *loxP* sites to resolve the selectable marker cassette in multiple gene deletions is highly undesirable, as it can lead to (large) genomic inversions and/or rearrangements (14, 15, 57).

Although the Cre-lox system as described here was used in *L. plantarum* WCFS1, it can easily be adapted to other Gram-positive bacteria, including other lactic acid bacteria such as *Lactococcus lactis* and *Streptococcus thermophilus*. The only prerequisite to use this system is the availability of a method to establish transient Cre expression. For example, a temperature-

sensitive vector such as pG⁺host (40) can be used for this purpose by sub-cloning of the P₁₁₄₄-Cre expression cassette present in pNZ5348. Applicability of this approach in *L. lactis* has already been established in our laboratory (R. Brooijmans, personal communication). More advanced alternative possibilities could also be employed, including for example strictly controlled Cre expression from a permanently present plasmid or from a chromosomal locus, or expression of Cre from a plasmid in which Cre or the origin of replication are flanked by *lox* sites to ensure cessation of Cre activity before construction of additional mutations in the same genetic background. Analogously, the mutagenesis vector pNZ5319 could be modified by replacement of the pACYC184 replicon by a temperature-sensitive replicon (pG⁺host-derived) to facilitate mutagenesis in bacterial species that display a low transformation frequency that eliminates the suicide mutagenesis strategy employed here. These relatively simple modifications allow functional implementation of the Cre-*lox*-based mutagenesis system in a range of other Gram-positive or Gram-negative bacteria, and exemplify the broad applicability of the system presented here.

In conclusion, the multiple gene deletion system for Gram-positive bacteria presented here allows effective, standardized double cross-over mutant constructions that can be introduced in a single genetic background by a simple repetitive procedure. The use of mutant *lox* sites for Cre-mediated selectable-marker removal enables stable multi-locus mutants that are suitable for subsequent rounds of mutagenesis.

REFERENCES

1. **Abremski, K., R. Hoess, and N. Sternberg.** 1983. Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. *Cell* **32**:1301-11.
2. **Abuin, A., and A. Bradley.** 1996. Recycling selectable markers in mouse embryonic stem cells. *Mol. Cell Biol.* **16**:1851-6.
3. **Adams, D. E., J. B. Bliska, and N. R. Cozzarelli.** 1992. Cre-*lox* recombination in *Escherichia coli* cells. Mechanistic differences from the *in vitro* reaction. *J. Mol. Biol.* **226**:661-73.
4. **Albert, H., E. C. Dale, E. Lee, and D. W. Ow.** 1995. Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. *Plant J.* **7**:649-59.
5. **Arakawa, H., D. Lodygin, and J. M. Buerstedde.** 2001. Mutant *loxP* vectors for selectable marker recycle and conditional knock-outs. *BMC Biotechnol.* **1**:7.
6. **Bhowmik, T., L. Fernandez, and J. L. Steele.** 1993. Gene replacement in *Lactobacillus helveticus*. *J. Bacteriol.* **175**:6341-4.
7. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513-23.
8. **Bron, P. A.** 2004. Ph.D. thesis. The molecular response of *Lactobacillus plantarum* to intestinal passage and conditions. Wageningen University, Wageningen, The Netherlands.

9. **Bron, P. A., M. G. Benchimol, J. Lambert, E. Palumbo, M. Deghorain, J. Delcour, W. M. De Vos, M. Kleerebezem, and P. Hols.** 2002. Use of the *alr* gene as a food-grade selection marker in lactic acid bacteria. *Appl. Environ. Microbiol.* **68**:5663-70.
10. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. *J. Bacteriol.* **186**:5721-9.
11. **Bron, P. A., S. M. Hoffer, I. I. van Swam, W. M. De Vos, and M. Kleerebezem.** 2004. Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. *Appl. Environ. Microbiol.* **70**:310-7.
12. **Bruno-Barcena, J. M., M. A. Azcarate-Peril, T. R. Klaenhammer, and H. M. Hassan.** 2005. Marker-free chromosomal integration of the manganese superoxide dismutase gene (*sodA*) from *Streptococcus thermophilus* into *Lactobacillus gasseri*. *FEMS Microbiol. Lett.* **246**:91-101.
13. **Camilli, A., D. T. Beattie, and J. J. Mekalanos.** 1994. Use of genetic recombination as a reporter of gene expression. *Proc. Natl. Acad. Sci. USA* **91**:2634-8.
14. **Campo, N., M. L. Daveran-Mingot, K. Leenhouts, P. Ritzenthaler, and P. Le Bourgeois.** 2002. Cre-loxP recombination system for large genome rearrangements in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **68**:2359-67.
15. **Campo, N., M. J. Dias, M. L. Daveran-Mingot, P. Ritzenthaler, and P. Le Bourgeois.** 2004. Chromosomal constraints in Gram-positive bacteria revealed by artificial inversions. *Mol. Microbiol.* **51**:511-22.
16. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.* **138**:179-207.
17. **de Ruyter, P. G., O. P. Kuipers, M. M. Beerthuyzen, I. van Alen-Boerrigter, and W. M. de Vos.** 1996. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. *J. Bacteriol.* **178**:3434-9.
18. **Dekker, R., Meer, R. van der, Olieman, C.** 1991. Sensitive pulsed amperometric detection of free and conjugated bile acids in combination with gradient reversed-phase HPLC. *Chromatographia* **31**:549-553.
19. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* **154**:1-9.
20. **Gilbertson, L.** 2003. Cre-lox recombination: Cre-ative tools for plant biotechnology. *Trends Biotechnol.* **21**:550-5.
21. **Goffin, P., F. Lorquet, M. Kleerebezem, and P. Hols.** 2004. Major role of NAD-dependent lactate dehydrogenases in aerobic lactate utilization in *Lactobacillus plantarum* during early stationary phase. *J. Bacteriol.* **186**:6661-6.
22. **Guldener, U., S. Heck, T. Fielder, J. Beinhauer, and J. H. Hegemann.** 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* **24**:2519-24.
23. **Hoess, R. H., and K. Abremski.** 1985. Mechanism of strand cleavage and exchange in the Cre-lox site-specific recombination system. *J. Mol. Biol.* **181**:351-62.
24. **Hols, P., T. Ferain, D. Garmyn, N. Bernard, and J. Delcour.** 1994. Use of homologous expression-secretion signals and vector-free stable chromosomal integration in engineering of *Lactobacillus plantarum* for alpha-amylase and levanase expression. *Appl. Environ. Microbiol.* **60**:1401-13.
25. **Josson, K., T. Scheirlinck, F. Michiels, C. Platteeuw, P. Stanssens, H. Joos, P. Dhaese, M. Zabeau, and J. Mahillon.** 1989. Characterization of a gram-positive broad-host-range plasmid isolated from *Lactobacillus hilgardii*. *Plasmid* **21**:9-20.

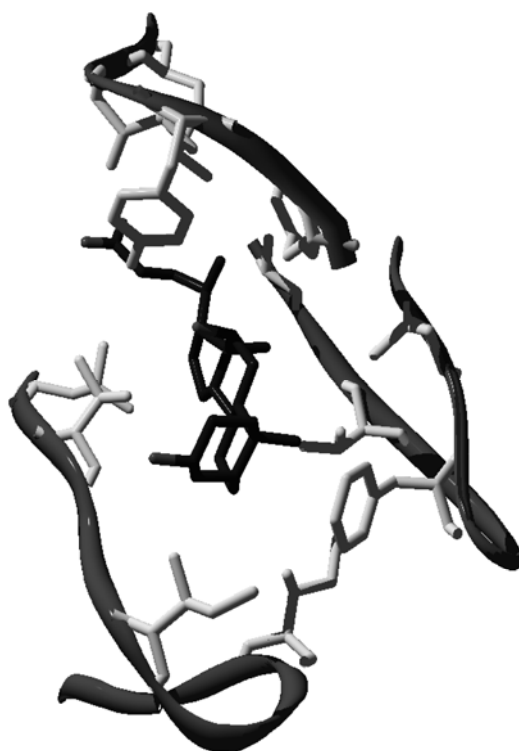
26. **Kijima, T., T. Osaki, K. Nishino, T. Kumagai, T. Funakoshi, H. Goto, I. Tachibana, Y. Tanio, and T. Kishimoto.** 1999. Application of the Cre recombinase/*loxP* system further enhances antitumor effects in cell type-specific gene therapy against carcinoembryonic antigen-producing cancer. *Cancer Res.* **59**:4906-11.
27. **Killmann, H., C. Herrmann, A. Torun, G. Jung, and V. Braun.** 2002. TonB of *Escherichia coli* activates FhuA through interaction with the beta-barrel. *Microbiology* **148**:3497-509.
28. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. USA* **100**:1990-5.
29. **Kolb, A. F.** 2001. Selection-marker-free modification of the murine beta-casein gene using a *lox2272* [correction of *lox2722*] site. *Anal. Biochem.* **290**:260-71.
30. **Kolb, A. F., and S. G. Siddell.** 1997. Genomic targeting of a bicistronic DNA fragment by Cre-mediated site-specific recombination. *Gene* **203**:209-16.
31. **Kristensen, C. S., L. Eberl, J. M. Sanchez-Romero, M. Givskov, S. Molin, and V. De Lorenzo.** 1995. Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid RP4. *J. Bacteriol.* **177**:52-8.
32. **Langer, S. J., A. P. Ghafouri, M. Byrd, and L. Leinwand.** 2002. A genetic screen identifies novel non-compatible *loxP* sites. *Nucleic Acids Res.* **30**:3067-77.
33. **Leenhouts, K., A. Bolhuis, G. Venema, and J. Kok.** 1998. Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. *Appl. Microbiol. Biotechnol.* **49**:417-23.
34. **Leenhouts, K. J., J. Kok, and G. Venema.** 1991. Replacement recombination in *Lactococcus lactis*. *J. Bacteriol.* **173**:4794-8.
35. **Leer, R. J., Christiaens, H., Verstraete, W., Peters, L., Posno, M., Pouwels, P. H.** 1993. Gene disruption in *Lactobacillus plantarum* strain 80 by site-specific recombination: isolation of a mutant strain deficient in conjugated bile salt hydrolase activity. *Mol. Gen. Genet.* **239**:269-272.
36. **Lin, M. Y., S. Harlander, and D. Savaiano.** 1996. Construction of an integrative food-grade cloning vector for *Lactobacillus acidophilus*. *Appl. Microbiol. Biotechnol.* **45**:484-9.
37. **Liu, S.** 2006. A simple method to generate chromosomal mutations in *Lactobacillus plantarum* strain TF103 to eliminate undesired fermentation products. *Appl. Biochem. Biotechnol.* **131**:854-63.
38. **Llanos, R. M., C. J. Harris, A. J. Hillier, and B. E. Davidson.** 1993. Identification of a novel operon in *Lactococcus lactis* encoding three enzymes for lactic acid synthesis: phosphofructokinase, pyruvate kinase, and lactate dehydrogenase. *J. Bacteriol.* **175**:2541-51.
39. **Llanos, R. M., A. J. Hillier, and B. E. Davidson.** 1992. Cloning, nucleotide sequence, expression, and chromosomal location of *ldh*, the gene encoding L-(+)-lactate dehydrogenase, from *Lactococcus lactis*. *J. Bacteriol.* **174**:6956-64.
40. **Maguin, E., P. Duwat, T. Hege, D. Ehrlich, and A. Gruss.** 1992. New thermosensitive plasmid for gram-positive bacteria. *J. Bacteriol.* **174**:5633-8.
41. **Marx, C. J., and M. E. Lidstrom.** 2002. Broad-host-range cre-lox system for antibiotic marker recycling in gram-negative bacteria. *Biotechniques* **33**:1062-7.
42. **Mills, D. A.** 2001. Mutagenesis in the post genomics era: tools for generating insertional mutations in the lactic acid bacteria. *Curr. Opin. Biotechnol.* **12**:503-9.

43. **Min Kim, J., J. Young Choi, M. Sun Kim, and S. Chang Kim.** 2004. *In vivo* excision and amplification of large human genomic segments using the Cre/*loxP*-and large T antigen/SV40 ori-mediated machinery. *J. Biotechnol.* **110**:227-33.
44. **Mlynarova, L., J. Libantova, L. Vrba, and J. P. Nap.** 2002. The promiscuity of heterospecific *lox* sites increases dramatically in the presence of palindromic DNA. *Gene* **296**:129-37.
45. **Nagy, A.** 2000. Cre recombinase: the universal reagent for genome tailoring. *Genesis* **26**:99-109.
46. **Palmeros, B., J. Wild, W. Szybalski, S. Le Borgne, G. Hernandez-Chavez, G. Gosset, F. Valle, and F. Bolivar.** 2000. A family of removable cassettes designed to obtain antibiotic-resistance-free genomic modifications of *Escherichia coli* and other bacteria. *Gene* **247**:255-64.
47. **Platteeuw, C., G. Simons, and W. M. de Vos.** 1994. Use of the *Escherichia coli* beta-glucuronidase (*gusA*) gene as a reporter gene for analyzing promoters in lactic acid bacteria. *Appl. Environ. Microbiol.* **60**:587-93.
48. **Polzin, K. M., and L. L. McKay.** 1992. Development of a lactococcal integration vector by using IS981 and a temperature-sensitive lactococcal replication region. *Appl. Environ. Microbiol.* **58**:476-84.
49. **Quenee, L., D. Lamotte, and B. Polack.** 2005. Combined *sacB*-based negative selection and Cre-*lox* antibiotic marker recycling for efficient gene deletion in *Pseudomonas aeruginosa*. *Biotechniques* **38**:63-7.
50. **Rose, R. E.** 1988. The nucleotide sequence of pACYC184. *Nucleic Acids Res.* **16**:355.
51. **Russell, W. M., and T. R. Klaenhammer.** 2001. Efficient system for directed integration into the *Lactobacillus acidophilus* and *Lactobacillus gasseri* chromosomes via homologous recombination. *Appl. Environ. Microbiol.* **67**:4361-4.
52. **Sadowski, P. D.** 1995. The FLP recombinase of the 2-microns plasmid of *Saccharomyces cerevisiae*. *Prog. Nucleic Acid Res. Mol. Biol.* **51**:53-91.
53. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
54. **Sauer, B.** 1987. Functional expression of the cre-*lox* site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **7**:2087-96.
55. **Sauer, B.** 1998. Inducible gene targeting in mice using the Cre/*lox* system. *Methods* **14**:381-92.
56. **Silvestroni, A., C. Connes, F. Sesma, G. S. De Giori, and J. C. Piard.** 2002. Characterization of the *mela* locus for alpha-galactosidase in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **68**:5464-71.
57. **Suzuki, N., H. Nonaka, Y. Tsuge, M. Inui, and H. Yukawa.** 2005. New multiple-deletion method for the *Corynebacterium glutamicum* genome, using a mutant *lox* sequence. *Appl. Environ. Microbiol.* **71**:8472-80.
58. **Tan, P. S., I. J. van Alen-Boerrigter, B. Poolman, R. J. Siezen, W. M. de Vos, and W. N. Konings.** 1992. Characterization of the *Lactococcus lactis pepN* gene encoding an aminopeptidase homologous to mammalian aminopeptidase N. *FEBS Lett.* **306**:9-16.
59. **van Alen-Boerrigter, I. J., R. Baankreis, and W. M. de Vos.** 1991. Characterization and overexpression of the *Lactococcus lactis pepN* gene and localization of its product, aminopeptidase N. *Appl. Environ. Microbiol.* **57**:2555-61.
60. **van Kranenburg, R., J. D. Marugg, S. van, II, N. J. Willem, and W. M. de Vos.** 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. *Mol. Microbiol.* **24**:387-97.

61. **Wertman, K. F., A. R. Wyman, and D. Botstein.** 1986. Host/vector interactions which affect the viability of recombinant phage lambda clones. *Gene* **49**:253-62.
62. **Wilson, J. W., D. H. Figurski, and C. A. Nickerson.** 2004. VEX-capture: a new technique that allows *in vivo* excision, cloning, and broad-host-range transfer of large bacterial genomic DNA segments. *J. Microbiol. Methods* **57**:297-308.
63. **Woodcock, D. M., P. J. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S. S. Smith, M. Z. Michael, and M. W. Graham.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res.* **17**:3469-78.
64. **Yanisch-Perron, C., Vieira, J. and Messing, J.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103-119.

Chapter 4.

Functional analysis of conjugated bile acid hydrolase family members in *Lactobacillus plantarum* WCFS1.



J. M. Lambert
R. S. Bongers
W. M. de Vos
M. Kleerebezem

4. Functional analysis of conjugated bile acid hydrolase family members in *Lactobacillus plantarum* WCFS1

Bile salts play an important role in the digestion of lipids in vertebrates, and are synthesized and conjugated to either glycine or taurine in the liver. Following secretion into the small intestine, intestinal microbes are capable of deconjugating the glycine or taurine from the bile salts using an enzyme called bile salt hydrolase (Bsh). Intestinal lactobacilli are regarded as major contributors to bile salt hydrolysis *in vivo*. Since the bile salt-hydrolyzing strain *Lactobacillus plantarum* WCFS1 was predicted to encode four *bsh*-genes (*bsh1*, *bsh2*, *bsh3*, and *bsh4*), the functionality of these *bsh* genes was explored using heterologous over-expression and multiple *bsh*-deletion strains. Thus, Bsh1 was shown to be responsible for the majority of Bsh activity in *L. plantarum* WCFS1. In addition, *bsh1* of *L. plantarum* WCFS1 was shown to be involved in conferring tolerance to specific bile salts (i.e., glycocholic acid). Northern blot analysis established that *bsh1*, *bsh2*, *bsh3*, and *bsh4* are all expressed in *L. plantarum* WCFS1 during the exponential growth phase. In biodiversity analysis, *bsh1* appeared to be the only *bsh* homologue variable among *L. plantarum* strains; furthermore, presence of *bsh1* correlated with presence of Bsh activity, suggesting that Bsh1 is commonly responsible for Bsh activity in *L. plantarum* strains. The fact that *bsh2*, *bsh3*, and *bsh4* genes appeared to be conserved among *L. plantarum* strains suggests an important role of these genes in the physiology and life-style of the species *L. plantarum*. Analysis of these additional *bsh*-like genes in *L. plantarum* WCFS1 suggests that they might encode penicillin acylase rather than Bsh activity. This could indicate their implication in conversion of substrates other than bile acids or penicillin-like compounds in the natural habitat.

INTRODUCTION

Bile salts play an essential role in lipid digestion in vertebrates. They act as a detergent that emulsifies and solubilises dietary lipids and lipid-soluble vitamins. In the liver, bile acids are synthesized and conjugated as an *N*-acyl amidate with the amino acid taurine or glycine before being excreted via the bile duct into the small intestine (Figure 1). Usually, species of the intestinal microbiota, including a number of lactobacilli, produce bile salt hydrolases that are able to deconjugate the amino acid moiety from the bile salts in the intestine (Figure 1). A strong correlation has been found between the habitat of a specific bacterial species or strain and Bsh activity (47), suggesting a relation between the capability to deconjugate bile salts and survival or persistence of bacteria under gastro-intestinal conditions. Furthermore, intestinal bile salt deconjugation is believed to play an important role in host physiology, as it is the gate-

keeping reaction in further oxidation and dehydroxylation steps of primary bile salts (as synthesized by the host) into secondary bile salts by intestinal bacteria. Notably, the production of secondary bile acids has been linked to various intestinal diseases such as the formation of gallstones and colon cancer (44).

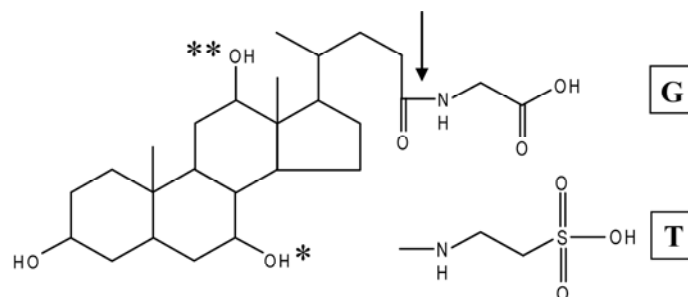


Figure 1. General structure of bile salts. As an example, the structure of glycocholic acid is shown. The bond that is hydrolysed by Bsh-activity is indicated by an arrow. G, glycine; T, taurine, * OH-group absent in glycodeoxycholic acid; ** OH-group absent in glycochenodeoxycholic acid.

According to the enzyme classification system, bile salt hydrolase (Bsh; EC3.5.1.24) belongs to the category of enzymes that act on carbon-nitrogen bonds in linear amides, other than peptide bonds. Among others, this enzyme category includes members of the β -lactam acylase family such as penicillin and cephalosporin acylases (EC3.5.1.11), and ceramidases (EC3.5.1.23). Although Bsh shares significant sequence homology with some of the enzymes in the EC3.5.1.- group and the type of bond that is cleaved is identical, the type of substrates that can be converted by the various enzymes is quite heterogeneous, and may vary significantly in molecule size and hydrophobicity.

Bsh activity has been found in a wide variety of mostly Gram-positive species (for review, see (3)), including *Bifidobacterium* (22, 25, 48), *Clostridium* (21, 26, 27, 33), *Enterococcus* (18), *Listeria* (4), and *Lactobacillus* (2, 9-11, 17, 23, 32, 34, 42), with the exception of the Gram-negative species *Bacteroides* (33, 46). The *Lactobacillus plantarum* WCFS1 genome (28) was predicted to contain four related genes annotated as *bsh1* to *bsh4* that are spread throughout the genome (28). However, functional analysis of a *L. plantarum* WCFS1 *bsh1* mutant suggested that *bsh1* encodes for the majority of Bsh activity produced by this strain (32).

Here, we present the functional analysis of *bsh1*, *bsh2*, *bsh3*, and *bsh4* of *L. plantarum* WCFS1. To investigate the predicted function of these genes, each of the four *bsh* genes was overexpressed in the Bsh-deficient *Lactococcus lactis*. In addition, single, double, triple, and quadruple *bsh* knockout mutants of *L. plantarum* WCFS1 were constructed to evaluate the contribution of the individual *bsh* genes to hydrolysis of and/or tolerance to various substrates,

including bile salts, penicillin V, and acyl-homoserine lactones. Furthermore, the evolutionary conservation of *bsh*-homologs was investigated in several strains of the species *L. plantarum* using complete genome hybridisation (CGH) (37). These results indicated that *bsh2*, *bsh3*, and *bsh4* appear to be conserved among *L. plantarum* strains, suggesting an important physiological role. In addition, *bsh1* presence appeared to be correlated to Bsh activity of *L. plantarum* strains.

MATERIALS AND METHODS

Bacterial strains, plasmids and primers. The bacterial strains, plasmids and primers used in this study and their relevant features are listed in Table 1 and 2. *L. plantarum* WCFS1 (28) and *bsh*-mutant derivatives were grown at 37°C in MRS broth (Difco, West Molesey, United Kingdom) without aeration. The heterologous, nisin controlled expression (NICE) host *L. lactis* NZ9000 and its parental strain MG1363 that was used as an intermediate cloning host for NICE overexpression constructs (35) were grown at 30°C in M17 broth (Oxoid, Hampshire, United Kingdom) supplemented with 0.5 % glucose (w/v; G-M17), without aeration. *Escherichia coli* strains DH5 α (54) and MC1061 (8, 53) were used as intermediate cloning host for *L. plantarum* mutagenesis constructs and pCR-Blunt constructs, respectively, and were grown at 37°C on TY broth (24), with aeration. When appropriate, antibiotics were added to the media. For *L. plantarum*, 10 μ g/ml chloramphenicol and 10 μ g/ml (in liquid medium) or 30 μ g/ml (on solid medium) erythromycin were used. For *L. lactis*, 10 μ g/ml chloramphenicol was used. For *E. coli*, 10 μ g/ml chloramphenicol and 250 μ g/ml erythromycin were used.

DNA and protein manipulations. Plasmid DNA was isolated from *E. coli* on a small scale using the alkaline-lysis method (5). Large-scale plasmid DNA isolations were performed using Jetstar columns as recommended by the manufacturer (Genomed GmbH, Bad Oberhausen, Germany). Purification of DNA fragments from agarose gels was performed using the Wizard SV gel and PCR clean-up system (Promega, Leiden, The Netherlands). DNA isolation and transformation of *L. plantarum* and *L. lactis* was performed as described previously (14, 32). For DNA manipulations in *E. coli*, protein extraction, and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), standard procedures were employed (45). Restriction endonucleases, *Taq*, *Pfx* and *Pwo* DNA polymerases, T4 DNA ligase, and Klenow enzyme were used as prescribed by the manufacturers (Promega, Leiden, The Netherlands, and Boehringer, Mannheim, Germany). Primers were obtained from Genset Oligos (Paris, France).

Functional analysis of conjugated bile acid hydrolase family members

Table 1. Strains and plasmids used in this study.

Material	Relevant feature(s) ^a	Reference
Strains		
<i>L. plantarum</i>		
WCFS1	Single colony isolate of NCIMB8826	(28)
NZ5305	<i>bsh1</i> deletion derivative of WCFS1 ($\Delta bsh1$)	(32)
NZ5307	<i>bsh2</i> deletion derivative of WCFS1 ($\Delta bsh2$)	This work
NZ5309	<i>bsh3</i> deletion derivative of WCFS1 ($\Delta bsh3$)	This work
NZ5311	<i>bsh4</i> deletion derivative of WCFS1 ($\Delta bsh4$)	This work
NZ5313	<i>bsh1</i> deletion derivative of NZ5307 ($\Delta bsh1$, $\Delta bsh2$)	This work
NZ5315	<i>bsh4</i> deletion derivative of NZ5309 ($\Delta bsh3$, $\Delta bsh4$)	This work
NZ5324	<i>bsh3</i> deletion derivative of NZ5313 ($\Delta bsh1$, $\Delta bsh2$, $\Delta bsh3$)	This work
NZ5326	<i>bsh4</i> deletion derivative of NZ5313 ($\Delta bsh1$, $\Delta bsh2$, $\Delta bsh4$)	This work
NZ5328	<i>bsh1</i> deletion derivative of NZ5315 ($\Delta bsh1$, $\Delta bsh3$, $\Delta bsh4$)	This work
NZ5330	<i>bsh2</i> deletion derivative of NZ5315 ($\Delta bsh2$, $\Delta bsh3$, $\Delta bsh4$)	This work
NZ5332	<i>bsh3</i> deletion derivative of NZ5326 ($\Delta bsh1$, $\Delta bsh2$, $\Delta bsh3$, $\Delta bsh4$)	This work
<i>L. lactis</i>		
MG1363	Used as cloning host for NICE expression vectors; plasmid-free derivative of NCDO 712	(20)
NZ9000	Used for heterologous expression using the NICE system; derivative of MG1363 with an insertion of the <i>nisR</i> , <i>K</i> genes into the <i>pepN</i> gene (<i>pepN::nisRK</i>)	(30)
Strains		
<i>E. coli</i>		
DH5 α	Used as cloning host for <i>L. plantarum</i> mutagenesis vectors; F ⁻ , ϕ 80dlacZ Δ M15, Δ (lacZYA-argF)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (r _k ⁻ , m _k ⁺), <i>phoA</i> , <i>supE44</i> , λ , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	(54)
MC1061	Used as cloning host for pCR-Blunt vectors; F ⁻ <i>araD139</i> Δ (<i>ara-leu</i>)7696 <i>galE15</i> <i>galK16</i> Δ (lac)X74 <i>rpsL</i> (<i>Str</i> ^r) <i>hsdR2</i> (r _k ⁻ m _k ⁺) <i>mcrA</i> <i>mcrB1</i>	(8, 53)
Plasmids		
pCR-Blunt	Kan ^R , commercial cloning vector for blunt-end PCR products	Invitrogen
pNZ5306	Cm ^R , pNZ8048 derivative; translational fusion of <i>bsh1</i> to the nisin promoter	This work
pNZ5307	Cm ^R , pNZ8150 derivative; translational fusion of <i>bsh2</i> to the nisin promoter	This work
pNZ5308	Cm ^R , pNZ8048 derivative; translational fusion of <i>bsh3</i> to the nisin promoter	This work
pNZ5309	Cm ^R , pNZ8048 derivative; translational fusion of <i>bsh4</i> to the nisin promoter	This work
pNZ5319	Cm ^R Em ^R , standard mutagenesis vector	(32)
pNZ5325	Cm ^R Em ^R , pNZ5319 derivative for <i>bsh1</i> mutagenesis	(32)
pNZ5329	Cm ^R Em ^R , pNZ5319 derivative for <i>bsh2</i> mutagenesis	This work
pNZ5332	Cm ^R Em ^R , pNZ5319 derivative for <i>bsh3</i> mutagenesis	This work
pNZ5336	Cm ^R Em ^R , pNZ5319 derivative for <i>bsh4</i> mutagenesis	This work
pNZ5348	Em ^R , expression of <i>cre</i> from a plasmid that is unstable in lactobacilli	(32)
pNZ8048	Cm ^R , NcoI site used for translational fusions to the nisin promoter	(30)
pNZ8150	Cm ^R , ScaI site used for translational fusions to the nisin promoter	(35)

^a Kan^R, kanamycin resistant; Cm^R, chloramphenicol resistant; Em^R, erythromycin resistant.

Table 2. Primers used in this study.

Primers	5' to 3' sequence
106a	gttcgataaagaatgaggatggc
107a	ttatcgcaagtatctcaaattgcg
85	gttttttctagtccaagctcaca ggattactagacatgtgtactgcc
87	gccgactgtactttcggatcct ggattactagacatgtgtactgcc
Bsh1F	ggattactagacatgtgtactgcc
Bsh1fr1F	gattaagtttgcaggacatggag
Bsh1intF	cgggattaaattttgcagg
Bsh1R	gccagccattggaacttactctg
Bsh2contrR	gagttactgctttctgaaagtc
Bsh2F2	ccctttacatgtgcactagtctaac
Bsh2fr1intF	catggaattgtcatatgatgtcg
Bsh2intF	tcatgcaatttaccagcc
Bsh2ko-down	cgatttgatcggcgggcagg
Bsh2ko-up	ctgagcgcataccaacttag
Bsh2kofr1F	catggcaccaatataccccgcctc
Bsh2kofr1R	tagactagtgcacatacaaatcgctcc
Bsh2kofr2F	ttgccagcggccattaagactttcag
Bsh2kofr2R	gctatgcaaccggtgcattgaaactg
Bsh2R	caagacgggtagccgtcactc
Bsh2seqR	cgagcgaattaaccttagc
Bsh3F	gtgaaacacatgtgtactagttaac
Bsh3fr1intF	gtaatccaaatgatgtggtcgat
Bsh3intF	tatttctcgggacaagc
Bsh3ko-down	cttgtcagttgcataagctcgc
Bsh3ko-up	ggtcgctgattatcaaagtagc
Bsh3kofr1NheF	aaaaccccgtagcgcgcagtccttggcgatgatggccg
Bsh3kofr1R	taaactagtacacatgggttcacatcc
Bsh3kofr2F	ttccggttagcaaactaagttggaatata
Bsh3kofr2R	gcaaataagtgaccaataaagtagcttacg
Bsh3R	gaaagtgcattctgcctag
Bsh4F2	ccctttacatgtgtaccagcttaac
Bsh4fr1intF	gctgttttgacacgcagatcatc
Bsh4intF	acgccgttgaatacg
Bsh4ko-down	gagaagcagtttggtcgtcag
Bsh4ko-up	ccttgacagttgccttaggatc
Bsh4kofr1F	cgggaatccgcacacaagttgcc
Bsh4kofr1R	taagctggtacacatggcgcggc
Bsh4kofr2F2	tttctgccgattgaaactaaaaaatc
Bsh4kofr2SalR	tctctcgtcgaccctaaacgctgtgggaggtgctg
Bsh4R	ggtcggaatgcagcgcaagctg

RNA isolation and Northern blotting. For RNA isolation, an overnight culture of *L. plantarum* WCFS1 was diluted 50-fold in 50 ml of fresh MRS medium, with or without the addition of 0.05 % (w/v) porcine bile (Sigma, Zwijndrecht, the Netherlands), and grown to an OD₆₀₀ of 1. Subsequently, 3 volumes of quench buffer were added (60% methanol, 66.7 mM HEPES, pH6.5, -40 °C) (43) . The cells were immediately pelleted by centrifugation at 4500 rpm

for 10 minutes (Megafuge 1.0R, Heraeus, Hanau, Germany), resuspended in 750 µl of ice-cold TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and mechanically disrupted (FastPrep FP120, Qbiogene, Illkirch, Cedex, France) in the presence of 0.8 g of zirconium beads (Biospec Products, Bartlesville, OK, USA), 0.18 g of Macaloid (Kronos Titan GmbH, Leverkusen, Germany), 50 µl of 10% SDS, and 500 µl of phenol. Subsequently, the RNA was purified from the upper, aqueous phase of the cell extract by phenol-chloroform extraction, precipitated with absolute ethanol, washed with 70% ethanol (45), and resuspended in 50 µl of MQ water. Northern blot analysis was performed as described earlier (45) using total RNA. As a probe for *bsh1*, *bsh2*, *bsh3*, and *bsh4*, a PCR amplification product of a large part of the gene (0.7 to 0.8 kb) was used, which was amplified with *Taq* polymerase, using *L. plantarum* WCFS1 total DNA as a template in combination with the primer sets bsh1intF/bsh1R, bsh2intF/bsh2seqR, bsh3intF/bsh3R, and bsh4intF/bsh4R, respectively (Table 2). Cross-hybridization of the individual probes with the other *bsh* sequences of *L. plantarum* WCFS1 was checked using dot-blot of 0, 0.2, 0.8, 3.0, 12.5, and 50 ng of PCR amplification products encompassing *bsh1*, *bsh2*, *bsh3*, or *bsh4*, respectively (amplified using *Taq* polymerase, WCFS1 total DNA, and primer sets bsh1F/bshR, bsh2F/bsh2seqR, bsh3F/bsh3R, and bsh4F/bsh4R, respectively [Table 2]).

Construction of *bsh* over-expression strains. For over-expression of *bsh1*, a DNA fragment containing the complete *bsh1* gene was amplified by PCR, using *Pfx* polymerase with *L. plantarum* WCSF1 genomic DNA as a template and primers bsh1F and bsh1R. The resulting amplicon was cloned into pCR-Blunt (Invitrogen, Breda, the Netherlands). Subsequently, the *bsh1* gene was recovered from the resulting plasmid as a 1.1 kb AflII-HindIII fragment and cloned downstream of and translationally fused to the *nisA* promoter in NcoI-HindIII digested pNZ8048 (35). The resulting nisin-controlled *bsh1* expression plasmid was designated pNZ5306.

A DNA fragment containing *bsh2* was amplified by PCR using *Pfx* polymerase with *L. plantarum* genomic DNA as a template and primers bsh2F2 and bsh2R. The amplicon obtained was digested with HindIII, and the resulting 1.1 kb fragment was cloned downstream of and translationally fused to the *nisA* promoter in pNZ8150 (35), digested with ScaI and HindIII, resulting in the *bsh2*-overexpression vector pNZ5307.

The over-expression plasmid for *bsh3* was constructed analogous to the *bsh1* plasmid pNZ5306. A *bsh3*-containing PCR amplicon (using *Pfx* polymerase, *L. plantarum* WCFS1 genomic DNA as a template, and primers bsh3F and bsh3R) was initially cloned in pCR-blunt (Invitrogen, Breda, the Netherlands), and a 1.1 kb fragment containing *bsh3* was sub cloned in pNZ8048 following the same cloning strategy as employed with *bsh1*, yielding pNZ5308 that contains the *bsh3* gene under control of the *nisA* transcription and translation signals.

Finally, a *bsh4* containing DNA fragment was amplified by PCR using *Pfx* polymerase with *L. plantarum* WCFS1 genomic DNA as a template and primers *bsh4F2* and *bsh4R*. The amplicon obtained was cloned into the pCR-Blunt vector. The *bsh4* gene was recovered from the resulting plasmid by digestion with *KpnI* and *ApaLI*, followed by partial digestion with *AflIII* and cloning of the 1.1 kb, *bsh4* containing fragment downstream of and translationally fused to the *nisA* promoter in *NcoI*-*KpnI* digested pNZ8048, yielding the *bsh4* overexpression construct pNZ5309.

For over-expression studies of the *bsh* genes in *L. lactis* using the NICE system, pNZ5306, pNZ5307, pNZ5308, and pNZ5309 were transformed to *L. lactis* NZ9000.

Construction of *bsh* deletion mutant strains. For the construction of deletion derivatives of *L. plantarum* WCFS1 that lack one or more of the *bsh* genes, the previously reported Cre-*lox*-based system for multiple gene deletions was used (32). The *bsh1*-deletion vector pNZ5325 and *bsh1*-deletion strain NZ5305 were constructed previously (32) (Table 1). The *bsh2*-mutagenesis vector pNZ5329 (Table 1) was constructed by successive cloning of the PCR-amplified 1.0 kb 5'- and 3'-chromosomal flanking regions of *bsh2* (lp_0067) (using *Pfx* polymerase, *L. plantarum* WCFS1 genomic DNA as a template, and the primer sets *bsh2kofr1F* / *bsh2kofr1R* and *bsh2kofr2F* / *bsh2kofr2R*, respectively [Table 2]) into the *SwaI* and *Ecl136II* restriction site of pNZ5319 (Table 1), respectively (32). Analogously, the *bsh3*- mutagenesis vector pNZ5332 (Table 1) was constructed by successive cloning of the PCR-amplified 1.0 kb 5'- and 3'-chromosomal flanking regions of *bsh3* (lp_3362) (using *Pfx* polymerase, *L. plantarum* WCFS1 genomic DNA as a template, and primer sets *bsh3kofr1NheF* / *bsh3kofr1R* and *bsh3kofr2F* / *bsh3kofr2R*, respectively [Table 2]) into the *PmeI* and *Ecl136II* restriction site of pNZ5319 (Table 1), respectively (32). Finally, the *bsh4*- mutagenesis vector pNZ5336 (Table 1) was constructed by successive cloning of the PCR-amplified 1.0 and 0.9 kb 5'- and 3'-chromosomal flanking regions of *bsh4* (lp_2572) (using *Pfx* polymerase, *L. plantarum* WCFS1 genomic DNA as a template, and primer sets *bsh4kofr1F* / *bsh4kofr1R* and *bsh4kofr2F2* / *bsh4kofr2SalR*, respectively [Table 2]) into the *SwaI* and *Ecl136II* restriction site of pNZ5319 (Table 1), respectively (32). For all *bsh*-deletion constructs, the sequences of the cloned PCR-amplified regions were verified by double strand sequence analysis (BaseClear, Roosendaal, The Netherlands).

The *bsh*-deletion strains NZ5305 ($\Delta bsh1$), NZ5307 ($\Delta bsh2$), NZ5309 ($\Delta bsh3$), NZ5311 ($\Delta bsh4$), NZ5313 ($\Delta bsh1$, $\Delta bsh2$), NZ5315 ($\Delta bsh3$, $\Delta bsh4$), NZ5324 ($\Delta bsh1$, $\Delta bsh2$, $\Delta bsh3$), NZ5326 ($\Delta bsh1$, $\Delta bsh2$, $\Delta bsh4$), NZ5328 ($\Delta bsh1$, $\Delta bsh3$, $\Delta bsh4$), NZ5330 ($\Delta bsh2$, $\Delta bsh3$, $\Delta bsh4$), NZ5332 ($\Delta bsh1$, $\Delta bsh2$, $\Delta bsh3$, $\Delta bsh4$) were constructed as described previously (32) (Table 1 and Figure 2). Briefly, the desired *bsh*-deletion vector was transformed to *L. plantarum* WCFS1 or one of its mutant derivatives by electroporation (32) and double

cross-over gene replacement mutants, in which the target gene was replaced by a *lox66*-P₃₂-*cat*-*lox71* cassette, were selected based on their Cm^R, Em^S phenotype. Correct integration of the *lox66*-P₃₂-*cat*-*lox71* cassette into the genome was confirmed by PCR amplification of the flanking regions of the integrated *lox66*-P₃₂-*cat*-*lox71* cassette, using primers annealing uniquely to genomic sequences combined with the mutagenesis vector-specific primers 85 and 87 that annealed to the P₃₂-*cat* region, respectively (i.e., primers 106a / 85 and 87 / 107a for *bsh1* replacement, *bsh2*ko-up / 85 and 87 / *bsh2*ko-down for *bsh2* replacement, *bsh3*ko-up / 85 and 87 / *bsh3*ko-down for *bsh3* replacement, and *bsh4*ko-up / 85 and 87 / *bsh4*ko-down for *bsh4* replacement [Table 2]). Subsequently, the *lox66*-P₃₂-*cat*-*lox71* cassette was removed from the genome by transient expression of the Cre recombinase enzyme from a plasmid that is unstable in lactobacilli (pNZ5348), leading to a stable deletion of the *bsh* gene, as described earlier (32). The Cre-*lox*-based mutagenesis system has specifically been designed to allow subsequent rounds of mutagenesis within a single genetic background, by using single nucleotide mutant *lox* sites (*lox66* and *lox71*) that after recombination lead to a double-mutant *lox* (*lox72*) recombination site that is not recognized by Cre in subsequent rounds of mutagenesis (32). The *L. plantarum* *bsh*-deletion mutant strains were checked by PCR, amplifying each of the four (mutated) *bsh* loci, using *bsh1*fr1F / *bsh1*R (yielding a 1.4 kb and 0.5 kb product for the wild type and mutated locus, respectively), *bsh2*fr1intF / *bsh2*contrR (yielding a 1.4 kb and 0.4 kb product for the wild type and mutated locus, respectively), *bsh3*fr1intF / *bsh3*R (yielding a 1.4 kb and 0.5 kb product for the wild type and mutated locus, respectively), *bsh4*fr1intF / *bsh4*R (yielding a 1.2 kb and 0.3 kb product for the wild type and mutated locus, respectively) for *bsh1*, *bsh2*, *bsh3*, and *bsh4*, respectively (Figure 3). Using this system, *L. plantarum* WCFS1 derivatives were constructed that have stable deletion mutations in one, two, three or all four of the *bsh* genes (see above and Table 1).

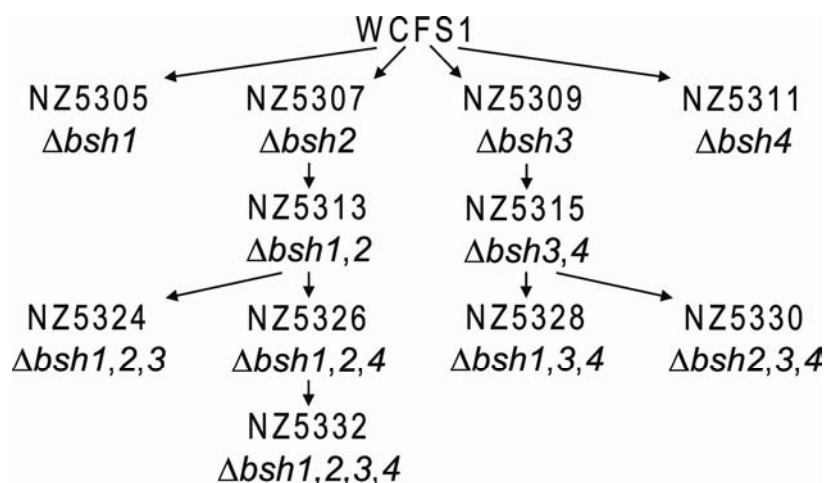


Figure 2. Schematic overview of phylogenetic relation of the *L. plantarum* *bsh* mutants that were constructed in this work.

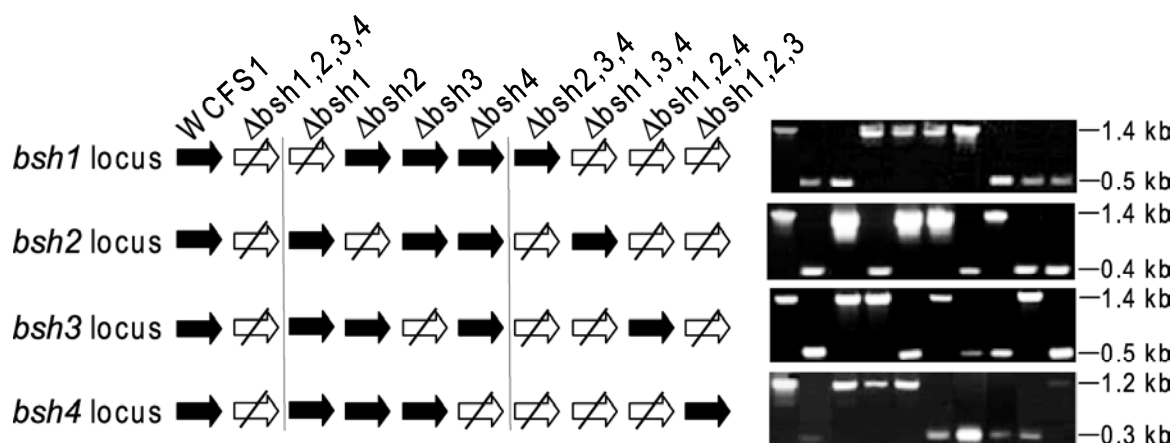


Figure 3. Schematic overview of the *bsh*-mutants made in *L. plantarum* WCFS1 (shown as a reference) and correspondingly, PCR-analysis encompassing the *bsh* loci of the mutants. Sizes of the PCR-products are indicated. $\Delta bsh1, 2, 3, 4$: NZ5332; $\Delta bsh1$: NZ5305; $\Delta bsh2$: NZ5307; $\Delta bsh3$: NZ5309; $\Delta bsh4$: NZ5311; $\Delta bsh2, 3, 4$: NZ5330; $\Delta bsh1, 3, 4$: NZ5328; $\Delta bsh1, 2, 4$: NZ5326; $\Delta bsh1, 2, 3$: NZ5324.

HPLC assay of Bsh activity. To determine Bsh activity of *L. plantarum* strains, an overnight culture was inoculated 1:10 in fresh MRS medium and cells were either grown to an OD₆₀₀ of 3 or grown overnight. Cells were harvested by centrifugation for 10 minutes at 4500 rpm at room temperature (Megafuge 1.0R, Heraeus, Hanau, Germany) and resuspended in MRS medium to an OD₆₀₀ of 100.

Overexpression of individual *bsh* genes in *L. lactis* using the nisin controlled expression system (NICE) was performed using established protocols (12). In short, overnight cultures of the *L. lactis* *bsh* over-expression strains were subcultured (1:50) in fresh G-M17 medium and grown to an OD₆₀₀ of 0.5. Subsequently, 1 ng/ml nisin (Sigma, Zwijndrecht, the Netherlands) was added to these cultures and growth was continued for 2 h. Cells were harvested by centrifugation for 10 minutes at 4500 rpm at room temperature (Megafuge 1.0R, Heraeus, Hanau, Germany). Cell pellets were resuspended to a final OD₆₀₀ of 200 in 55 mM sodium acetate buffer pH6.5 containing 1 mM DTT and 1 gram of zirconium beads (51) and mechanically disrupted (FastPrep FP120, Qbiogene, Illkirch, Cedex, France). Following centrifugation, cell-free extracts were used immediately. Protein concentration in cell extracts was determined as described previously (6).

Conversion of 1 mmol/l of the bile salts glycocholic acid (GC), glycodeoxycholic acid (GDC), glycochenodeoxycholic acid (GCDC), taurocholic acid (TC), taurodeoxycholic acid (TDC), and taurochenodeoxycholic acid (TCDC) (Sigma, Zwijndrecht, the Netherlands) or Fischer rat bile (courtesy of Wageningen University, Wageningen, The Netherlands) by intact cells of *L. plantarum* or cell-free extract of *L. lactis* *bsh*

over-expression strains was determined at 37°C by HPLC as described previously (15). Separations were carried out with a reversed-phase resin-based column (PLRP-S, 5 µm, 300 Å, 250 x 4.6 mm I. D., Polymer laboratories, Shropshire, UK) and matching pre-column. Bile salts were detected using a pulsed amperometric detector (EG&G Princeton applied research, Princeton, NJ), equipped with a gold working electrode and a reference electrode (Ag/AgCl). Chromatograms were analyzed and integrated using the Chromeleon program (Dionex, Sunnyvale, USA) and Bsh activity was determined based on the disappearance of the conjugated bile salts used as a substrate.

Alternative acylase functionality. Cell-free extracts of *bsh*-overexpressing *L. lactis* strains in physiological salt (following procedures as described above) were analyzed for alternative acylase activity (Table 3). To determine penicillin, ampicillin, cephalosporin, acyl-homoserine lactone, and phenylacetyl glycine acylase activity, 5 volumes of the cell-free extract were mixed with 5 volumes of 100 mM sodium acetate buffer pH5 containing 1 mM DTT and 1 volume of 100 mM of penicillin V, penicillin G, ampicillin, cephalosporin C, ketocaproyl-homoserine lactone, oxooctanoyl-homoserine lactone, or phenylacetyl glycine and incubated overnight at 37°C. To stop the reaction, 35 volumes of 285 mM sodium acetate buffer pH4 were added. Free amino groups resulting from enzymatic conversion of the substrate were detected by the addition of 5 volumes of 10 mg/ml fluorescamine in acetone, centrifugation for 10 minutes at 4000 rpm at room temperature (TechnoSpin R, Sorval Instruments, Chandler Wilmington, USA), and measurement of fluorescence in the supernatant (excitation at 360 nm, emission at 465 nm; GENios F129004, Tecan Benelux, Giessen, the Netherlands). As a positive control, purified penicillin acylase and purified end-products of the acylase reactions (6-aminopenicillanic acid, 7-aminocephalosporanic acid, homoserine lactone, and glycine) were used (Sigma, Zwijndrecht, the Netherlands). Furthermore, acylase activity for 6-nitro-3-(phenylacetamido) benzoic acid (NIPAB; Sigma, Zwijndrecht, the Netherlands), which is a commonly used chromogenic substrate for assaying of penicillin G acylase activity, was determined as described earlier (1, 31). Briefly, 1 volume of cell-free extract was mixed with 9 volumes of 100 mM sodium acetate buffer containing 1 mM DTT and 2.5 mM NIPAB. Acylation of NIPAB at 25°C was determined by the increase of absorption at 405 nm, which was followed for 30 minutes and measured overnight. As a positive control, purified penicillin amidase of *E. coli* was used (Sigma, Zwijndrecht, The Netherlands).

Table 3. Overview of the substrates tested to investigate alternative functionality of the *bsh* genes of *L. plantarum* WCFS1.

Substrate	Comments
Penicillin G	Acylated by penicillin acylase
Penicillin V	Acylated by penicillin acylase
Ketocaproyl-homoserine lactone	Involved in quorum sensing
Oxo-octanoyl-homoserine lactone	Involved in quorum sensing
NIPAB	Used for determination of penicillin acylase activity
Phenylacetyl-glycine	Phenylalanine metabolism, acylated by Pva* of <i>E. coli</i>
Ampicillin	β -lactam
Cephalosporin C	β -lactam

*Pva: penicillin V acylase.

Bile salt and penicillin V tolerance. To evaluate the tolerance of *L. plantarum* WCFS1 and its *bsh*-mutant derivatives to bile salts and penicillins, an overnight culture was inoculated 1:20 into fresh MRS medium containing 0 to 30% ox gall (w/v), 0 to 0.4 % (w/v) GDC, 0 to 10% (w/v) TDC, and 0 to 14 μ g/ml penicillin V. Growth was followed for 16 hours by measurement of the OD₆₀₀ at 37°C at intervals of 15 min. (Spectra Max Plus 384, Molecular Devices, Sunnyvale, USA).

***bsh* diversity in *L. plantarum* strains.** The genomic diversity of *L. plantarum* strains (299; 299v; CIP102359; CIP104440; CIP104441; CIP104448; CIP104450; CIP104451; CIP104452; LP85-2; NCIMB12120; SF2A35B) has previously been investigated using strain WCFS1 derived DNA micro arrays (37). This genomic genotyping database allowed the evaluation of the presence and/or absence of homologues of the four genes that have initially been annotated in the *L. plantarum* WCFS1 genome as *bsh* (*bsh1*, *bsh2*, *bsh3*, and *bsh4*). Following statistical analysis, a positive cut-off P-value of $1.e^{-5}$ was used for presence calling of *bsh*-homologs.

Detection of Bsh activity in *L. plantarum* strains. The presence of Bsh activity in different *L. plantarum* strains (299; 299v; CIP102359; CIP104440; CIP104441; CIP104448; CIP104450; CIP104451; CIP104452; LP85-2; NCIMB12120, and SF2A35B) was detected using a bile salt plate-assay, as described earlier (11). Briefly, overnight cultures of *L. plantarum* strains were transferred to solid MRS medium with or without 0.5 % (w/v) of the bile salt taurodeoxycholic acid and incubated anaerobically for 48 hours at 37° C. Bsh-active strains were recognized by the formation of opaque white colonies when grown in presence of taurodeoxycholic acid, which is due to the precipitation of deconjugated bile salt forms.

RESULTS

Expression of *bsh* genes. *L. plantarum* WCFS1 contains four related *bsh* genes (28). The expression of these genes was studied by Northern blotting during exponential growth phase in the presence or absence of porcine bile (Figure 4A). The probes used for Northern blotting were specific for each of the individual *bsh* genes and did not show cross-hybridisation (Figure 4B). The Northern blot analysis established that *bsh1*, *bsh2*, *bsh3*, and *bsh4* are all expressed in *L. plantarum* WCFS1 during the exponential growth phase. The estimated sizes of the transcripts corresponded with those predicted for monocistronic transcription of *bsh1*, *bsh2*, *bsh3*, and *bsh4* (1.2 kb for *bsh1*, *bsh2*, and *bsh3*; 1.1 kb for *bsh4*). Incubation of *L. plantarum* WCFS1 grown in liquid medium with 0.5% (w/v) porcine bile did not induce significant expression of any of the *bsh* genes (Figure 4).

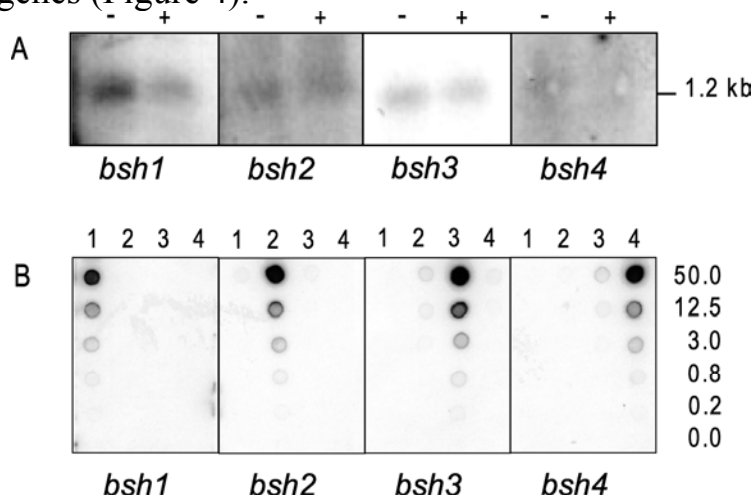


Figure 4 (A) Northern blot analysis of *bsh1*, *bsh2*, *bsh3*, and *bsh4* of *L. plantarum* WCFS1. -, growth of *L. plantarum* WCFS1 without porcine bile; +, growth of *L. plantarum* WCFS1 with 0.5% (w/v) porcine bile. (B) Determination of cross-hybridization of *bsh*-gene-specific probes. 1, 2, 3, 4: Hybridizing DNA of *bsh1*, *bsh2*, *bsh3*, or *bsh4*, respectively; 0.0, 0.2, 0.8, 3.0, 12.5, 50.0; amount of hybridizing DNA used for dot blotting (ng); *bsh1*, *bsh2*, *bsh3*, *bsh4*: *bsh*-gene-specific probe used for dot blotting.

Contribution to Bsh activity of individual Bsh proteins. Since all *bsh* genes of *L. plantarum* WCFS1 appeared to be expressed during exponential growth, the contribution of the individual *bsh* genes to the total Bsh activity was determined. For this purpose, heterologous *bsh* over-expression strains of *L. lactis* were established. Furthermore, a set of single and multiple *bsh* deletion derivatives of *L. plantarum* WCFS1 was constructed (Table 1).

For heterologous over-expression using the NICE-system (35), vectors pNZ5306, pNZ5307, pNZ5308, or pNZ5309, containing *bsh1*, *bsh2*, *bsh3*, or *bsh4* genes, respectively, translationally coupled to the nisin-inducible *nisA* promoter were constructed and transformed to the Bsh-deficient bacterium *L. lactis* NZ9000. Only following nisin induction, over-expression protein products could be detected by SDS-PAGE for Bsh2 and Bsh3 (Figure 5A) at their expected molecular mass of 38 and 36 kDa, respectively. In contrast, the *bsh1*

and *bsh4*-derived protein product could not be detected on protein gels, suggesting that these proteins are produced in much lower amounts in this heterologous host. HPLC-based Bsh-activity assays (15) using taurine- and glycine-conjugated bile salts of C, DC, and CDC as a substrate (Figure 1) revealed that Bsh1, Bsh3, and Bsh4 are capable of bile salt deconjugation (Figure 5A), confirming the functional expression of both Bsh1 and 4, despite the lack of detection of the corresponding protein product by SDS-PAGE. Bsh1 and Bsh3 displayed a strong preference for glycine-conjugated bile salts as compared to taurine-conjugated bile salts, while Bsh4 appeared to exclusively convert glycine-conjugated bile salts. Moreover, consistent differential substrate specificity of Bsh1, Bsh3 and Bsh4 was observed, with a substrate preference diminishing from DC to CDC and to C-conjugated bile salts (Figure 5B).

Contrary to the other Bsh proteins, no Bsh-activity could be detected for Bsh2 with any of the substrates tested. Notably, the suggestion that *bsh1* encodes the majority of Bsh activity in *L. plantarum* WCFS1 (32) was confirmed by these experiments, since the lactococcal strain expressing this gene displayed the highest level of Bsh activity, while no visible over-expression protein product could be detected by SDS-PAGE.

In parallel, a set of combinatorial *bsh*-mutant derivatives (Figure 3, Table 1 and Figure 2) of *L. plantarum* WCFS1 was constructed using a Cre-*lox*-based mutation system that allows the effective deletion of multiple genes in a single genetic background (32). The *bsh*-mutant derivatives were checked by PCR, amplifying each of the four *bsh* loci (Figure 3). Growth appeared to be unaffected in these mutants as compared to the parental strain, indicating that the deleted genes were not necessary for growth under normal laboratory conditions (data not shown). Moreover, no difference in colony morphology was observed between wild-type and mutant strains on MRS growth medium.

Bsh activity was analysed using the HPLC-based assay (15) with GDC as a substrate for the triple and quadruple *bsh*-mutant strains (NZ5324, NZ5326, NZ5328, NZ5330, and NZ5332, respectively); Figure 5C. These experiments revealed that cells harbouring an intact *bsh1* gene-copy (NZ5330; $\Delta bsh2$, $\Delta bsh3$, $\Delta bsh4$) displayed Bsh activity levels that were comparable to the wild-type strain, confirming previous studies identifying this gene as the major Bsh-encoding gene in *L. plantarum* (32). Moreover, Bsh1-dependent Bsh activity declined drastically (about 40 times) when comparing cells harvested during logarithmic growth phase with those that were obtained from the stationary phase of growth (Figure 5C), indicating growth phase-dependent expression of the *bsh1* gene. Notably, in all triple-mutant and *bsh1*-deficient strains (NZ5324, NZ5326, NZ5328), a small but detectable amount of bile salt hydrolysis was found (Figure 5C) that appeared to be *bsh2*-, *bsh3*-, or *bsh4*-independent. This finding was further confirmed by the presence of background Bsh-activity in the quadruple *bsh*-mutant strain NZ5332 (Figure 5C).

Functional analysis of conjugated bile acid hydrolase family members

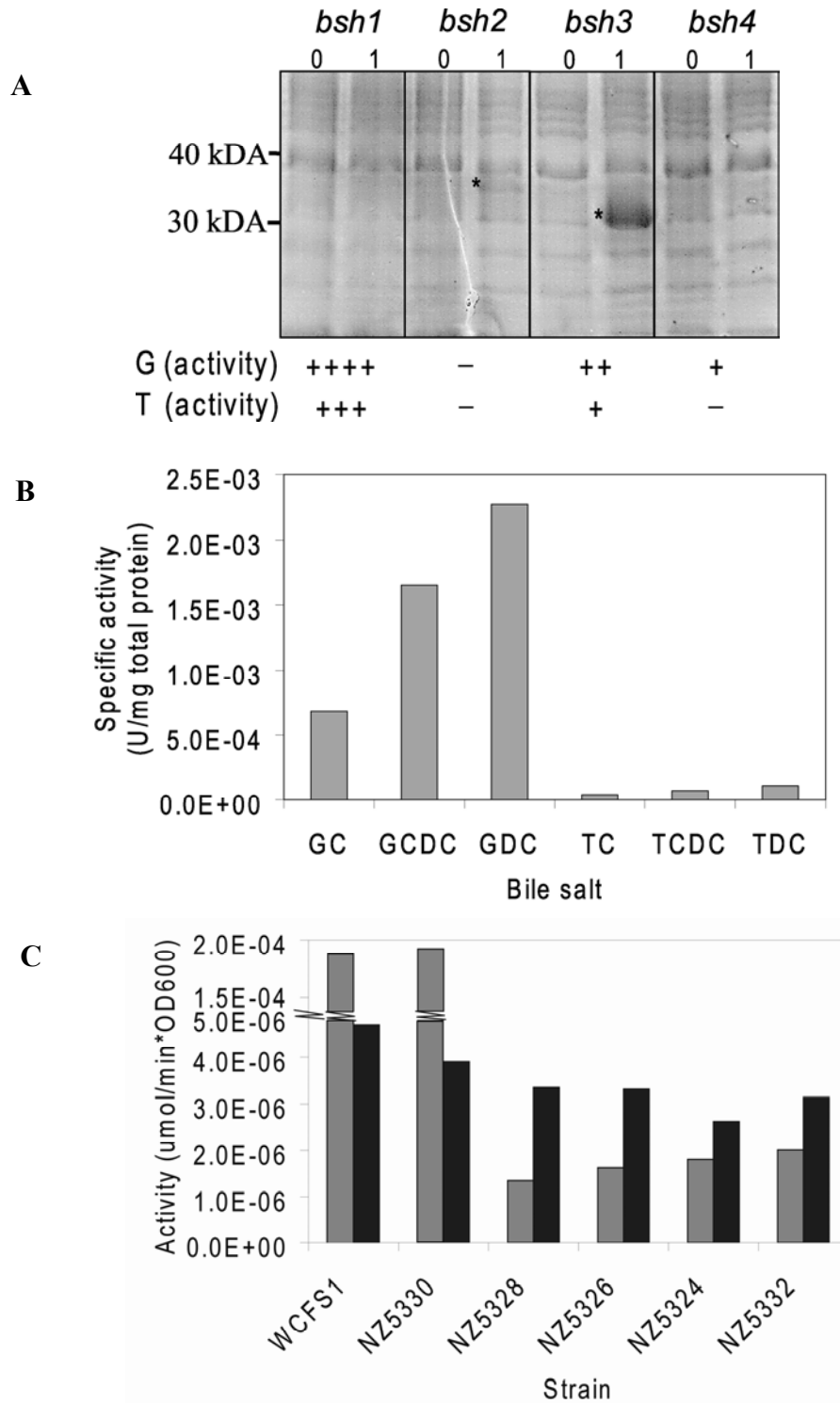


Figure 5. (A) Protein gel and activity of heterologous overexpression products of *bsh1*, *bsh2*, *h3*, and *bsh4* using the NICE system (35) in *L. lactis*. The predicted size of the Bsh1, Bsh2, Bsh3, and Bsh4 proteins was 37, 38, 36, and 36 kDa, respectively. For Bsh2 overexpression, no Bsh activity was detected. Bsh3 and Bsh4 were capable of marginal Bsh-activity; with no activity found for Bsh4 for taurine-conjugated substrates. In contrast, Bsh1 over-expression yielded major Bsh-activity. 0, no nisin induction; 1, induction with 1 ng/ml nisin; *, indication of protein band differentially expressed following nisin induction. (B) Substrate preference of the Bsh proteins. For all heterologous Bsh-overexpression strains, substrate preference diminished from GDC to TC. (Continued on next page).

As an example, the substrate preference of the Bsh1-overexpression strain is shown. (C) glycodeoxycholic acid deconjugation activity of *L. plantarum* WCFS1 and *bsh*-mutant derivatives at logarithmic and stationary growth phase. Grey bars represent the Bsh activity in logarithmic growth phase; black bars represent the Bsh activity in stationary phase. NZ5330: $\Delta bsh2$, $\Delta bsh3$, $\Delta bsh4$ phenotype; NZ5328: $\Delta bsh1$, $\Delta bsh3$, $\Delta bsh4$ phenotype; NZ5326: $\Delta bsh1$, $\Delta bsh2$, $\Delta bsh4$ phenotype; NZ5324: $\Delta bsh1$, $\Delta bsh2$, $\Delta bsh3$ phenotype; NZ5332: $\Delta bsh1$, $\Delta bsh2$, $\Delta bsh3$, $\Delta bsh4$ phenotype.

Nevertheless, based on these experiments, differential substrate specificity for the *bsh2*, *bsh3*, and *bsh4* gene products could not be excluded. To determine the contribution of the individual *bsh* genes to Bsh activity on complex bile, the Bsh activity of the triple and quadruple *bsh*-mutant derivatives (NZ5324, NZ5326, NZ5328, NZ5330, and NZ5332, respectively) of *L. plantarum* WCFS1 on male Fischer rat bile was determined. Clearly, *L. plantarum* WCFS1 was capable of hydrolysing most of the bile salts present in rat bile (Figure 6). Consistent with the results obtained with the pure bile salt substrate GDC, Bsh1 appeared to be responsible for the Bsh activity of *L. plantarum* WCFS1 on the complex rat bile substrate (Figure 6), while in *bsh1* deficient strains a low but consistent level of background activity was found that appeared to be independent of *bsh2*, *bsh3* or *bsh4* functionality.

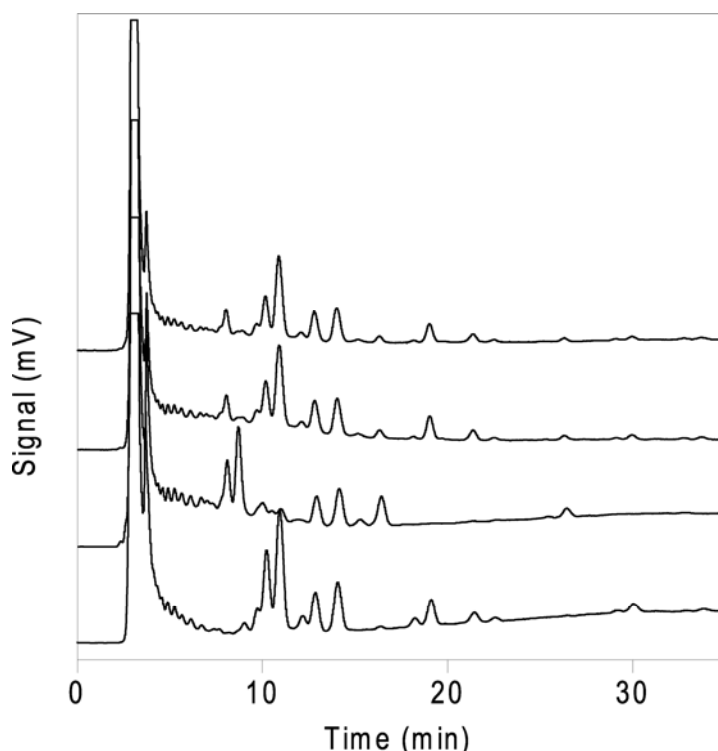


Figure 6. Schematic representation of the HPLC-profiles of rat bile salts before and after incubation with *L. plantarum* WCFS1 and *bsh*-mutant strains. From bottom to top, the HPLC-profiles are depicted of native Fischer rat bile, Fischer rat bile incubated with WCFS1, NZ5326 ($\Delta bsh1$, $\Delta bsh2$, $\Delta bsh4$) and NZ5332 ($\Delta bsh1$, $\Delta bsh2$, $\Delta bsh3$, $\Delta bsh4$). Clearly, most rat bile salts can be deconjugated by WCFS1, as can be seen by the shifting of the conjugated bile salt peaks in the chromatogram towards deconjugated bile salt peaks, whereas in strains lacking *bsh1*, no *bsh2*, *bsh3*, or *bsh4*-dependent bile salt deconjugation was found.

Alternative acylase activity. To determine alternative acylase activity of the Bsh proteins of *L. plantarum* WCFS1, a variety of alternative substrates was screened (Table 3). The Bsh proteins of *L. plantarum* WCFS1 share significant sequence homology with penicillin V acylase enzyme family-members. Furthermore, β -lactam acylases belong to the same category of proteins as penicillin V acylases, and are homologous to enzymes designated acyl-homoserine lactone acylase, which hydrolyse a similar chemical bond as β -lactam acylases and Bshs in acyl-homoserine lactones (Figure 7A). Therefore, the functionality of the different *bsh* gene-products of *L. plantarum* WCFS1 in the conversion of alternative substrates was screened by determination of the hydrolysis activity of over-expression products of the individual Bsh proteins using the NICE system (35) in *L. lactis* NZ9000 (using pNZ5306, pNZ5307, pNZ5308, and pNZ5309, respectively). Substrates tested were penicillin V, penicillin G, NIPAB (which is a commonly used substrate for spectrophotometric detection of penicillin acylase activity), the β -lactams ampicillin and cephalosporin C, ketocaproyl-homoserine lactone, oxooctanoyl-homoserine lactone, and phenylacetyl-glycine, which is a molecule that is involved in phenylalanine metabolism and thereby readily available to *L. plantarum*, and is cleaved by penicillin acylase of *E. coli* (50) (Table 3; Figure 7A). For NIPAB, ampicillin, cephalosporin C, and phenylacetyl-glycine, no activity could be detected in any of the Bsh-overexpression strains. However, *bsh3* (and to a lower extent *bsh2* and *bsh4*) over-expression resulted in an average of 4.3, 2.2 and 1.8 times increase in acylase activity of penicillin V, respectively, relative to the control strain NZ9000 in several independent experiments (Figure 7B). These findings clearly suggest a role as a penicillin acylase for *bsh3*, and possibly *bsh2* and *bsh4*. In addition, *bsh2*, *bsh3*, and *bsh4* over-expression strains all showed an increase in activity (on average 1.9 times) towards penicillin G, ketocaproyl- and oxooctanoyl-homoserine lactone relative to NZ9000, suggesting a broad range of enzyme specificity. Notably, the *bsh1* over-expression strain showed no significant activity towards penicillin V, penicillin G, ketocaproyl- or oxooctanoyl-homoserine lactone when compared to NZ9000, confirming the role of *bsh1* as a bonafide Bsh.

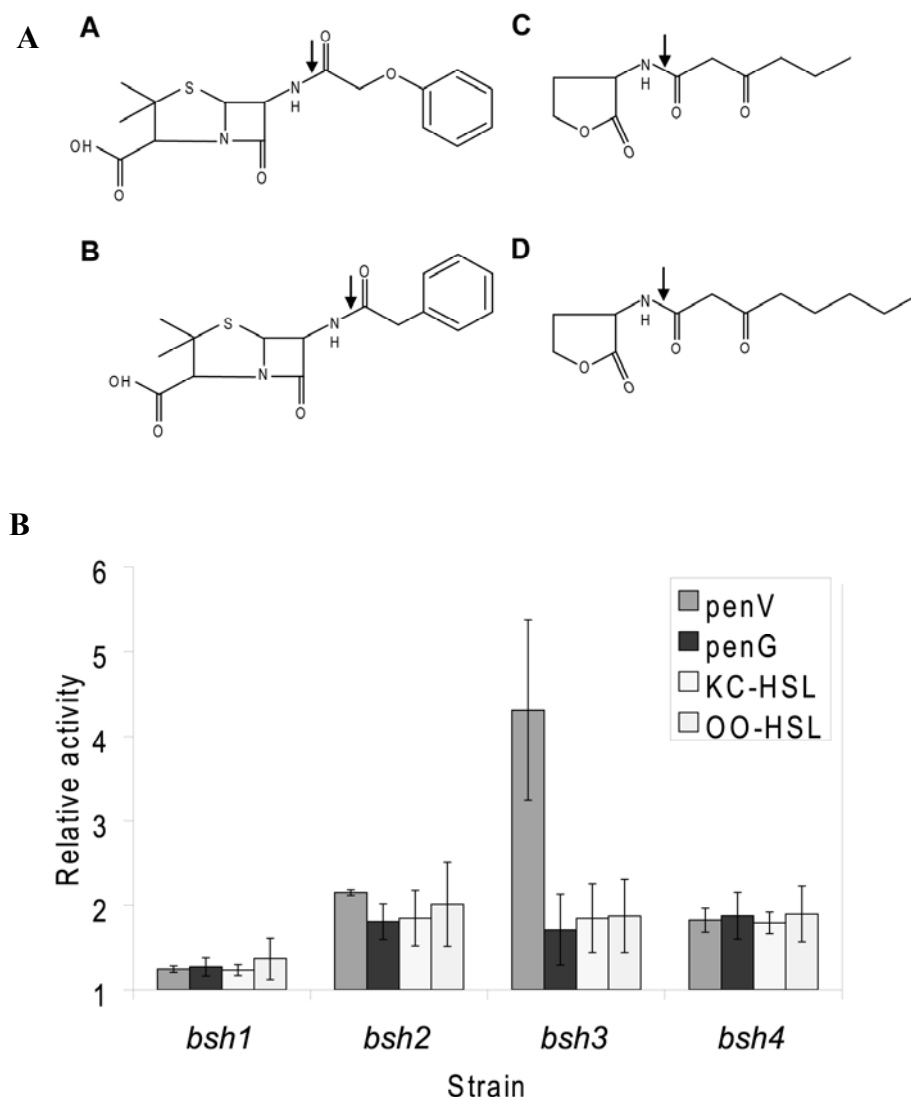


Figure 7. (A) Chemical structure of penicillin V (A), penicillin G (B), ketocaproyl-homoserine lactone (C), and oxooctanoyl-homoserine lactone (D). The acylation site is indicated with an arrow. (B) Fold change of activity of heterologous over-expression products of *bsh1*, *bsh2*, *bsh3*, and *bsh4* using the NICE system (35) in *L. lactis* NZ9000 relative to NZ9000 for penicillin V (penV), penicillin G (penG), ketocaproyl-homoserine lactone (KC-HSL) and oxooctanoyl-homoserine lactone (OO-HSL).

Bile salt and penicillin V tolerance. The contribution of the individual *bsh* genes to the tolerance of the bile salts TDC and GDC, ox-gall, and penicillin V (Figure 1 and Figure 7A) was determined using the triple and quadruple *L. plantarum* *bsh*-mutant strains (NZ5324, NZ5326, NZ5328, NZ5330, and NZ5332, respectively). To this end, growth in presence of a range of bile (salt) and penicillin concentrations was monitored spectrophotometrically.

L. plantarum was able to grow in the presence of up to 30% (w/v) of ox gall, while showing no significant differences in growth between the strains (data not shown). However, due to the high concentrations of ox gall used, measurements were severely hampered. In addition, *L. plantarum* was able to

grow in the presence of more than 14% (w/v) of TDC, where no significant differences were found for the strains used (data not shown). However, *L. plantarum* appeared to be remarkably more sensitive to GDC, with obvious differences between the strains (Figure 8).

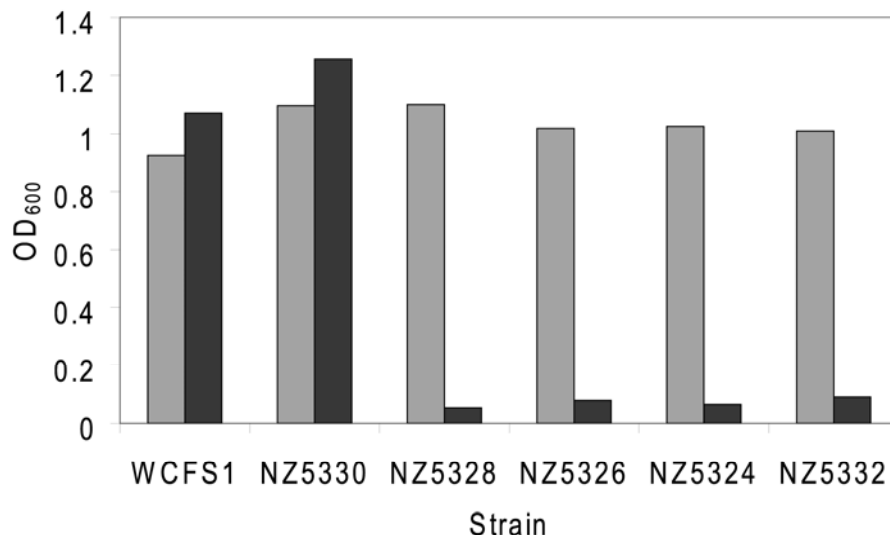


Figure 8. Representative result of GDC tolerance of *L. plantarum* WCFS1 wild type and *bsh* mutant derivatives, indicated by growth (OD₆₀₀) of the strains at 37°C after 7 hours in the presence of 0.1% (w/v) of GDC. NZ5330 ($\Delta bsh2$, $\Delta bsh3$, $\Delta bsh4$), NZ5328 ($\Delta bsh1$, $\Delta bsh3$, $\Delta bsh4$), NZ5326 ($\Delta bsh1$, $\Delta bsh2$, $\Delta bsh4$), NZ5324 ($\Delta bsh1$, $\Delta bsh2$, $\Delta bsh3$), NZ5332 ($\Delta bsh1$, $\Delta bsh2$, $\Delta bsh3$, $\Delta bsh4$).

The results clearly established that presence of *bsh1* in *L. plantarum* enhances GDC bile salt tolerance. Each of the *bsh1*-deficient derivatives displayed GDC-growth inhibition at a concentration as low as 0.1% (w/v) GDC. In contrast, strains containing an intact *bsh1*-gene were capable to sustain normal growth characteristics up to 0.5 to 0.7% (w/v) GDC. Analogous with the limited level of hydrolytic activity towards bile salts, *bsh2*, *bsh3*, and *bsh4* did not appear to contribute significantly to tolerance of GDC.

Furthermore, growth of *L. plantarum* was inhibited at the lowest concentration of penicillin V tested (0.3 µg/ml), with complete inhibition of growth in presence of 8 µg/ml penicillin V. However, no difference was found between WCFS1 and its *bsh*-mutant derivatives, indicating that none of the *bsh* genes appeared to influence penicillin V tolerance in *L. plantarum* under the conditions analyzed here.

Divergence of Bsh in *L. plantarum*. In order to further explore the functionality of the *bsh*-genes of *L. plantarum* the presence of homologues of the four *bsh* genes of *L. plantarum* WCFS1 in other *L. plantarum* strains was determined by analysis of *L. plantarum* genomic DNA samples on *L. plantarum* WCFS1-specific micro-arrays, as described earlier (37), using a positive cut-off P-value of $1.e^{-5}$ for *bsh* gene presence calls. These presence and absence calls were correlated with the experimentally determined capability of these strains to

hydrolyse bile salts, using a previously described Bsh plate-assay (11). Remarkably, in all *L. plantarum* strains, *bsh2*, *bsh3*, and *bsh4* generated very good probability scores for gene presence, suggesting that these genes are highly conserved among *L. plantarum* strains. In contrast, the presence of a gene homologous to *bsh1* appeared to vary among the strains analyzed. The *bsh1* appeared to be absent in four of the thirteen strains analyzed, which appeared to correlate relatively well with the absence of Bsh activity in these four strains. The remaining nine strains appeared to contain a *bsh1*-homologue as concluded from CGH analysis. Seven of these nine strains also displayed clearly detectable Bsh activity in the plate assay employed, while the residual two strains were not displaying this activity in this assay (Figure 9). Therefore, it is likely that Bsh activity is related to the presence of a *bsh1*-homologue in the species *L. plantarum*.

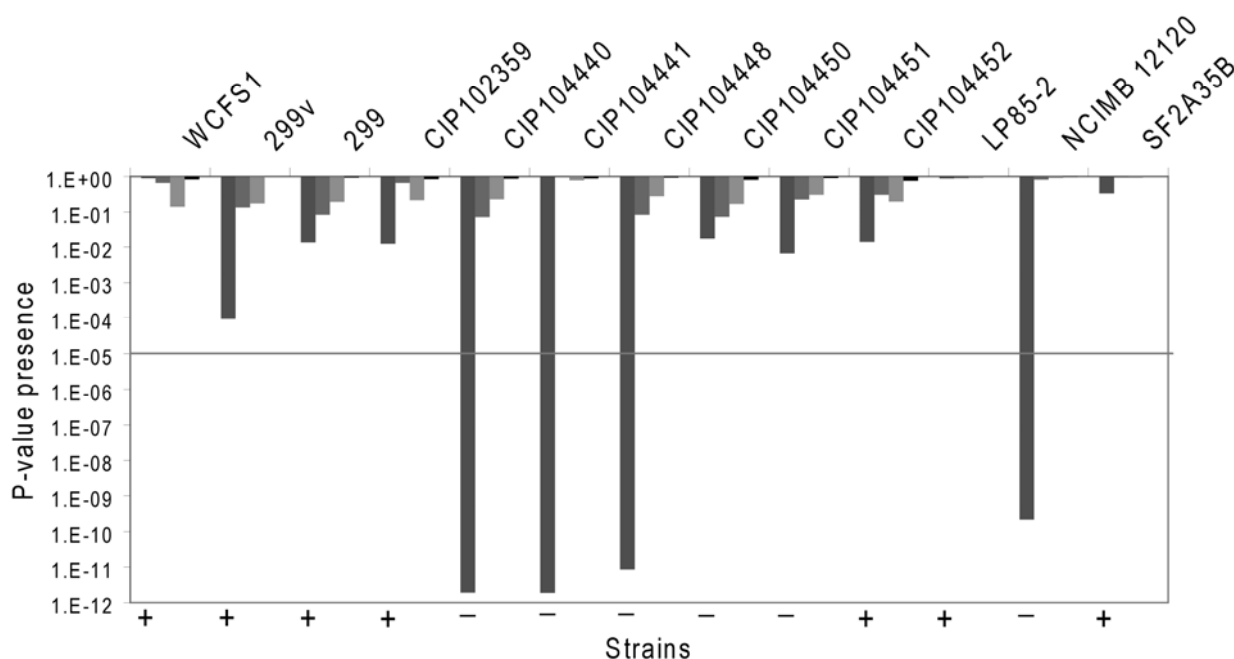


Figure 9. Diversity analysis of *bsh* genes in *L. plantarum* strains. Micro-array-based analysis of presence of *bsh1* to *bsh4* homologues in *L. plantarum* strains was correlated to the capability to hydrolyse the bile salt taurodeoxycholic acid. As a cut-off value for presence or absence calling of the *bsh* genes, a P-value of $1.e^{-5}$ was used. +, presence of Bsh activity; -, absence of Bsh activity.

DISCUSSION

Bile salt hydrolysis is a biologically important reaction in the intestinal tract, since it is the first step in bile salt biotransformations carried out by intestinal bacteria. The formation of secondary bile salts has a significant impact

on the physiology of the host, as exemplified by their implication in lowering of blood cholesterol levels (41), and various intestinal diseases such as the formation of gallstones and colon cancer (44). Furthermore, deconjugated bile salts were shown to induce mucin production by intestinal epithelial cells (29), possibly indicating irritation of the epithelial cells by the strong surface active properties of deconjugated bile salts.

Remarkably, in several strains (e.g. *L. johnsonii* 100-100 and NCC533, *L. acidophilus* NCFM, and *L. plantarum* WCFS1), the presence of more than one gene encoding a Bsh homologue has been predicted. In *L. plantarum* WCFS1, the sequences of the *bsh* genes share a higher level of similarity with *bsh* genes from other strains or species than with each other. For example, *bsh1* shares highest sequence similarity with the *bsh* genes of other *L. plantarum* strains and *Enterococcus faecalis*, whereas *bsh2* and *bsh4* share sequence similarity with the sequence annotated as *bsh* of *L. brevis*, and share no significant sequence similarity with any other organism of which the sequence is publicly available; in analogy, *bsh3* shares significant sequence similarity only with the sequence annotated as *bsh* of *L. sakei* and *L. brevis*. The *bsh* genes of *L. plantarum* WCFS1 may have been acquired via horizontal gene transfer, as has been suggested earlier for *L. johnsonii* (17). However, the overall conservation among *L. plantarum* strains of the *bsh2* to *bsh4* genes would indicate that this acquisition has occurred very early in the evolution of this species. Moreover, this high level of conservation also supports an important role of these genes in the physiology and life-style of the species *Lactobacillus plantarum*.

In line with the previous finding that *bsh1* is the major Bsh in *L. plantarum* WCFS1 (32), *bsh1* presence appeared to correlate with the capability to hydrolyse bile salts in eleven out of thirteen *L. plantarum* strains. For two strains, a Bsh1-homolog was concluded to be present, while the capability to hydrolyse taurodeoxycholic acid was not detected in these strains. This apparent inconsistency could have various reasons: 1. the presence of disruptive mutations in the *bsh1*-like gene detected by CGH analysis, 2. the gene detected encodes an intact Bsh enzyme but is not expressed under the conditions applied in the enzyme assay, 3. the enzyme encoded by the *bsh1*-homologue displays a more stringent substrate preference as compared to the Bsh1 of strain WCFS1 and fails to convert TDCA.

In *L. plantarum* WCFS1, the presence of *bsh1* correlated with GDC tolerance, but not with TDC tolerance. The capacity to hydrolyse bile salts has been found to be linked to bile tolerance in *L. plantarum* (13)} and several other bacteria, including *L. amylovorus* (23), *Listeria monocytogenes* (4)}, and *Bifidobacterium* (39). Thus, the preference of *L. plantarum* WCFS1 for deconjugation of glycine- over taurine-conjugated bile salts appears to be reflected in the differential tolerance towards GDC and TDC. This could be related to the higher toxicity of glycine-conjugated bile salts as compared to taurine-conjugated bile salts, leading to evolution of a preference of Bsh for

glycine-conjugated bile salts. Indeed, most Bshs show a preference for glycine-conjugated bile salts (3). Although the precise mechanism is unknown, bile salt hydrolysis could be of great importance for survival *in vivo*. Since deconjugated bile acids display reduced solubility as compared to their conjugated counterparts, especially at lower pH values, bile salt hydrolysis may lead to precipitation of the bile salts and thereby relieve stress levels caused by these surface active chemicals. Thereby, the capability to hydrolyse bile salts may contribute to the survival and persistence of bacterial strains in the intestinal tract, as has previously been shown in *Listeria monocytogenes* (16). Analogously, Bsh activity appears to be present in all lactobacilli isolated from the gastrointestinal environment (47). Notably, *L. plantarum* WCFS1 is capable of persisting in the mouse gastro-intestinal tract for 10 days (40) and has been shown to display relatively high survival and activity during transit of the human gastrointestinal tract (52).

All four of the *bsh* genes of *L. plantarum* WCFS1 appeared to be expressed during the exponential phase of growth as monocistronic transcripts (Figure 4). The expression of the *bsh*-genes did not appear to be induced as a consequence of exposure to porcine bile during growth in liquid media. In contrast, previous whole genome transcriptome studies have suggested that expression of *bsh1* was induced by porcine bile when cells are grown on solid media, while the expression of *bsh3* appeared to be repressed under these conditions (Bron et al. 2006). In the same study, no regulation of *bsh2* or *bsh4* by porcine bile was detected. Notably, these findings are in apparent agreement with the finding that *bsh1*, and to a lesser extent *bsh3*, are capable of bile salt hydrolysis. Nevertheless, the discrepancy in regulation of *bsh* expression may be due to the difference in growth conditions used (liquid vs. solid medium). To explain these different findings would require further investigation of regulation of expression of *bsh* as a function of culture conditions.

Heterologous over-expression of the *bsh* genes using the NICE-system in the Bsh-deficient host *L. lactis* NZ9000 confirmed that Bsh1 encodes a functional and bonafide Bsh, as was described earlier (32). In addition, Bsh3 and Bsh4 displayed bile salt hydrolysis activity, although with lower enzymatic efficiency as compared with Bsh1, which suggests that these enzymes could contribute to the overall Bsh activity displayed by *L. plantarum* WCFS1. Analogous to what has been described for most of the Bsh enzymes studied to date (26, 36, 48, 49), the Bsh1, Bsh3, and Bsh4 enzymes displayed a clear preference for glyco-conjugated over tauro-conjugated bile salts. Furthermore, the substrate preference of *bsh1*, *bsh3*, and *bsh4* decreased from deoxycholic to chenodeoxycholic and cholic acid as the steroid moiety of the conjugated bile salts (Figure 1 and Figure 5B), which has previously also been found for the Bsh activity of *L. buchneri* (38). This substrate preference is probably related to the positioning and presence or absence of hydroxyl-groups on the steroid moiety of

the bile salts (Figure 1), and concomitantly, the binding pocket properties of the enzyme.

Mutation analysis in *L. plantarum* WCFS1 confirmed that Bsh1 encodes the major Bsh. However, no contribution of Bsh2, Bsh3, or Bsh4 to the overall Bsh activity of *L. plantarum* WCFS1 could be detected, using either purified bile salt components or complex rat-bile salts as the substrate. This finding was in good agreement with the observation that the reduced bile salt hydrolysis activity found in *bsh1* single mutant derivative equals the level observed in a quadruple *bsh*-mutant derivative, which suggests that *bsh2*, *bsh3*, and *bsh4* do not contribute significantly to this enzyme activity in *L. plantarum*. Nevertheless, differential activity under specific conditions or highly selective activity towards specific bile salts substrates cannot be excluded for Bsh2, Bsh3, and/or Bsh4.

The CBAH family-members encoded by *L. plantarum* WCFS1 share significant sequence homology with penicillin V acylases, including the experimentally verified penicillin V acylase of *Listeria monocytogenes* EGDe (4). The sequence identity among these enzyme family members ranges from 30 to 36 % at the amino acid level. Furthermore, β -lactam acylases are homologous to enzymes designated acyl-homoserine lactone acylase, which hydrolyse a similar chemical bond as hydrolysed by β -lactam and Bshs, although present in a different class of molecules called acyl-homoserine lactones (Figure 7A) that play a key-role in quorum sensing-dependent gene regulation in Gram-negative bacteria (7). Screening for involvement of the *bsh* genes of *L. plantarum* WCFS1 in the conversion of penicillins, acyl-homoserine lactones, NIPAB, ampicillin, cephalosporin C, and phenylacetyl glycine showed that Bsh3, and to a lesser extent Bsh2 and Bsh4, were able to hydrolyse penicillin V. In addition, low-level acylase activity by Bsh2, Bsh3, and Bsh4 towards penicillin G, and both types of acyl-homoserine lactones tested was found, indicating broad substrate specificity of these enzymes. Acyl-homoserine lactones could have an important function in adhesion of bacteria to the epithelium of the intestinal tract. For example, the pathogen *Pseudomonas aeruginosa* was found to up-regulate PA-1 lectin/adhesin, an important virulence factor in this strain, in response to butanoyl-homoserine lactone in the environment (55). Therefore, bacteria that are capable of cleaving acyl-homoserine lactone could be of importance in preventing the adhesion of pathogens in the intestinal tract. Nevertheless, the low activity levels observed indicate that these substrates are not likely to represent the preferred substrate for *bsh2*, *bsh3*, or *bsh4*. Contrary to Bsh2, Bsh3, and Bsh4, Bsh1 displayed no detectable activity towards the alternative substrates tested, suggesting that Bsh1 displays a more narrow substrate specificity as compared to Bsh2, Bsh3, or Bsh4. Despite these results, no contribution of any of the *bsh* genes to penicillin V tolerance in *L. plantarum* could be detected in the *bsh*-deletion derivatives.

To date, the *in vivo* role of penicillin acylase remains unknown. Notably, the enzyme name appears to reflect primarily its industrial application rather than its natural substrate. Therefore, Bsh2, Bsh3 and Bsh4 may play a role in acylation of compounds other than the substrates tested here, such as additional phenylacetic acid derivatives, as suggested by the capability of penicillin acylases to cleave these substrates in addition to penicillins (50). Notably, phenylacetic acid derivatives would be available to *L. plantarum in vivo*, since they are formed by microbial activity on plant constituents, where *L. plantarum* was found to occur naturally (19). However, these substrates are not commercially available.

In conclusion, Bsh1 was found to be responsible for the majority of Bsh activity in *L. plantarum* WCFS1, and possibly in all *L. plantarum* strains. Computational analyses predicted three other Bsh-encoding genes to be present in *L. plantarum* WCFS1, while experimental evidence showed that the functionality of these genes is unclear, but possibly relates to acylase activity with penicillin-like chemicals as substrate. Notably, the conservation of *bsh2*, *bsh3*, and *bsh4* suggests an important but so far unknown role of these genes in the physiology and life-style of the species *L. plantarum*.

REFERENCES

1. **Alkema, W. B., R. Floris, and D. B. Janssen.** 1999. The use of chromogenic reference substrates for the kinetic analysis of penicillin acylases. *Anal. Biochem.* **275**:47-53.
2. **Bateup, J. M., McConnell, M. A., Jenkinson, H. F., Tannock, G. W.** 1995. Comparison of *Lactobacillus* strains with respect to bile salt hydrolase activity, colonization of the gastrointestinal tract, and growth rate of the murine host. *Applied and Environmental Microbiology* **61**:1147-1149.
3. **Begley, M., C. Hill, and C. G. Gahan.** 2006. Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol.* **72**:1729-38.
4. **Begley, M., R. D. Sleator, C. G. Gahan, and C. Hill.** 2005. Contribution of three bile-associated loci, *bsh*, *pva*, and *btlB*, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. *Infect. Immun.* **73**:894-904.
5. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513-23.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-54.
7. **Camilli, A., and B. L. Bassler.** 2006. Bacterial small-molecule signaling pathways. *Science* **311**:1113-6.
8. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.* **138**:179-207.
9. **Christiaens, H., Leer, R. J., Pouwels, P. H., Verstraete, W.** 1992. Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* by using a direct plate assay. *Applied and Environmental Microbiology* **58**:3792-3798.
10. **Corzo, G. a. G., S. E.** 1999. Bile salt hydrolase activity of three strains of *Lactobacillus acidophilus*. *Journal of Dairy Science* **82**:472-480.

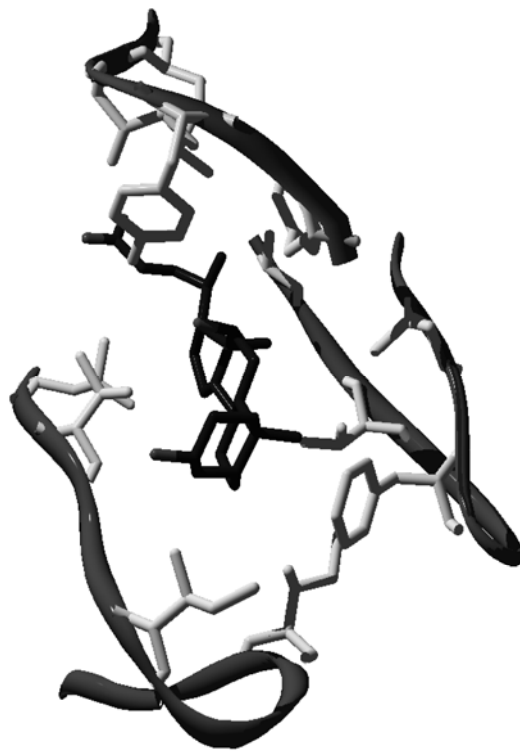
11. **Dashkevicz, M. P. a. F., S. D.** 1989. Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. *Applied and Environmental Microbiology* **55**:11-16.
12. **de Ruyter, P. G., O. P. Kuipers, M. M. Beerthuyzen, I. van Alen-Boerrigter, and W. M. de Vos.** 1996. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. *J. Bacteriol.* **178**:3434-9.
13. **De Smet, I., L. Van Hoorde, M. Vande Woestyne, H. Christiaens, and W. Verstraete.** 1995. Significance of bile salt hydrolytic activities of lactobacilli. *J. Appl. Bacteriol.* **79**:292-301.
14. **de Vos, W. M., P. Vos, H. de Haard, and I. Boerrigter.** 1989. Cloning and expression of the *Lactococcus lactis* subsp. cremoris SK11 gene encoding an extracellular serine proteinase. *Gene* **85**:169-76.
15. **Dekker, R., Meer, R. van der, Olieman, C.** 1991. Sensitive pulsed amperometric detection of free and conjugated bile acids in combination with gradient reversed-phase HPLC. *Chromatographia* **31**:549-553.
16. **Dussurget, O., D. Cabanes, P. Dehoux, M. Lecuit, C. Buchrieser, P. Glaser, and P. Cossart.** 2002. *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. *Mol. Microbiol.* **45**:1095-106.
17. **Elkins, C. A., Moser, S. A., Savage, D. C.** 2001. Genes encoding bile salt hydrolases and conjugated bile salt transporters in *Lactobacillus johnsonii* 100-100 and other *Lactobacillus* species. *Microbiology* **147**:3404-3412.
18. **Franz, C. M. A. P., Specht, I., Haberer, P., Holzapfel, W. H.** 2001. Bile salt hydrolase activity of enterococci isolated from food: screening and quantitative determination. *Journal of Food Protection* **64**:725-729.
19. **Gardner, N. J., T. Savard, P. Obermeier, G. Caldwell, and C. P. Champagne.** 2001. Selection and characterization of mixed starter cultures for lactic acid fermentation of carrot, cabbage, beet and onion vegetable mixtures. *Int. J. Food Microbiol.* **64**:261-75.
20. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* **154**:1-9.
21. **Gopal-Srivastava, R. a. H., P. B.** 1988. Purification and characterization of bile salt hydrolase from *Clostridium perfringens*. *Journal of Lipid Research* **29**:1079-1085.
22. **Grill, J., F. Schneider, J. Crociani, and J. Ballongue.** 1995. Purification and Characterization of Conjugated Bile Salt Hydrolase from *Bifidobacterium longum* BB536. *Appl. Environ. Microbiol.* **61**:2577-2582.
23. **Grill, J. P., Cayuela, C., Antoine, J. M., Schneider, F.** 2000. Isolation and characterization of a *Lactobacillus amylovorus* mutant depleted in conjugated bile salt hydrolase activity: relation between activity and bile salt resistance. *Journal of Applied Microbiology* **89**:553-563.
24. **Killmann, H., C. Herrmann, A. Torun, G. Jung, and V. Braun.** 2002. TonB of *Escherichia coli* activates FhuA through interaction with the beta-barrel. *Microbiology* **148**:3497-509.
25. **Kim, G. B., C. M. Miyamoto, E. A. Meighen, and B. H. Lee.** 2004. Cloning and characterization of the bile salt hydrolase genes (*bsh*) from *Bifidobacterium bifidum* strains. *Appl. Environ. Microbiol.* **70**:5603-12.
26. **Kirby, L. C., Klein, R. A., Coleman, J. P.** 1995. Continuous spectrophotometric assay of conjugated bile acid hydrolase. *Lipids* **30**:863-867.

27. **Kishinaka, M., A. Umeda, and S. Kuroki.** 1994. High concentrations of conjugated bile acids inhibit bacterial growth of *Clostridium perfringens* and induce its extracellular cholyglycine hydrolase. *Steroids* **59**:485-9.
28. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. U. S. A.* **100**:1990-5.
29. **Klinkspoor, J. H., R. Kuver, C. E. Savard, D. Oda, H. Azzouz, G. N. Tytgat, A. K. Groen, and S. P. Lee.** 1995. Model bile and bile salts accelerate mucin secretion by cultured dog gallbladder epithelial cells. *Gastroenterology* **109**:264-74.
30. **Kuipers, O. P., P. G. G. A. de Ruyter, M. Kleerebezem.** 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology* **64**:7.
31. **Kutzbach, C., and E. Rauenbusch.** 1974. Preparation and general properties of crystalline penicillin acylase from *Escherichia coli* ATCC 11 105. *Hoppe Seylers Z. Physiol. Chem.* **355**:45-53.
32. **Lambert, J. M., R. S. Bongers, and M. Kleerebezem.** 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **73**:1126-35.
33. **Masuda, N.** 1981. Deconjugation of bile salts by *Bacteroides* and *Clostridium*. *Microbiol. Immunol.* **25**:1-11.
34. **McAuliffe, O., R. J. Cano, and T. R. Klaenhammer.** 2005. Genetic analysis of two bile salt hydrolase activities in *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* **71**:4925-9.
35. **Mierau, I., and M. Kleerebezem.** 2005. 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Appl. Microbiol. Biotechnol.* **68**:705-17.
36. **Min Kim, J., J. Young Choi, M. Sun Kim, and S. Chang Kim.** 2004b. In vivo excision and amplification of large human genomic segments using the Cre/loxP-and large T antigen/SV40 ori-mediated machinery. *J. Biotechnol.* **110**:227-33.
37. **Molenaar, D., F. Bringel, F. H. Schuren, W. M. de Vos, R. J. Siezen, and M. Kleerebezem.** 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. *J. Bacteriol.* **187**:6119-27.
38. **Moser, S. A. a. S., D. C.** 2001. Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. *Applied and Environmental Microbiology* **67**:3476-3480.
39. **Noriega, L., I. Cuevas, A. Margolies, and C. G. de los Reyes-Gavilán.** 2006. Deconjugation and bile salts hydrolase activity by *Bifidobacterium* strains with acquired resistance to bile *International Dairy Journal* **16**:6.
40. **Pavan, S., P. Desreumaux, and A. Mercenier.** 2003. Use of mouse models to evaluate the persistence, safety, and immune modulation capacities of lactic acid bacteria. *Clin. Diagn. Lab. Immunol.* **10**:696-701.
41. **Pereira, D. I., and G. R. Gibson.** 2002. Effects of consumption of probiotics and prebiotics on serum lipid levels in humans. *Crit. Rev. Biochem. Mol. Biol.* **37**:259-81.
42. **Pereira, D. I., A. L. McCartney, and G. R. Gibson.** 2003. An in vitro study of the probiotic potential of a bile-salt-hydrolyzing *Lactobacillus fermentum* strain, and determination of its cholesterol-lowering properties. *Appl. Environ. Microbiol.* **69**:4743-52.
43. **Pieterse, B., R. H. Jellema, and M. J. van der Werf.** 2006. Quenching of microbial samples for increased reliability of microarray data. *J. Microbiol. Methods* **64**:207-16.

44. **Ridlon, J. M., D. J. Kang, and P. B. Hylemon.** 2006. Bile salt biotransformations by human intestinal bacteria. *J. Lipid Res.* **47**:241-59.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Stellwag, E. J., Hylemon, P.B.** 1976. Purification and characterization of bile salt hydrolase from *Bacteroides fragilis* subsp. *fragilis*. *Biochim. Biophys. Acta* **452**:165-76.
47. **Tanaka, H., Doesburg, K., Iwasaki, T., Mierau, I.** 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. *Journal of Dairy Science* **82**:2530-2535.
48. **Tanaka, H., Hashiba, H., Kok, J., Mierau, I.** 2000. Bile salt hydrolase of *Bifidobacterium longum* - biochemical and genetic characterization. *Applied and Environmental Microbiology* **66**:2502-2512.
49. **Taranto, M. P., F. Sesma, G. Font de Valdez.** 1999. Localization and primary characterization of bile salt hydrolase from *Lactobacillus reuteri*. *Biotechnology Letters* **21**:4.
50. **Valle, F., P. Balbas, E. Merino, and F. Bolivar.** 1991. The role of penicillin amidases in nature and in industry. *Trends Biochem. Sci.* **16**:36-40.
51. **van der Meer, J. R., J. Polman, M. M. Beerthuyzen, R. J. Siezen, O. P. Kuipers, and W. M. De Vos.** 1993. Characterization of the *Lactococcus lactis* nisin A operon genes *nisP*, encoding a subtilisin-like serine protease involved in precursor processing, and *nisR*, encoding a regulatory protein involved in nisin biosynthesis. *J. Bacteriol.* **175**:2578-88.
52. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. *Aliment. Pharmacol. Ther.* **14**:823-8.
53. **Wertman, K. F., A. R. Wyman, and D. Botstein.** 1986. Host/vector interactions which affect the viability of recombinant phage lambda clones. *Gene* **49**:253-62.
54. **Woodcock, D. M., P. J. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S. S. Smith, M. Z. Michael, and M. W. Graham.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res.* **17**:3469-78.
55. **Wu, L., C. Holbrook, O. Zaborina, E. Ploplys, F. Rocha, D. Pelham, E. Chang, M. Musch, and J. Alverdy.** 2003. *Pseudomonas aeruginosa* expresses a lethal virulence determinant, the PA-I lectin/adhesin, in the intestinal tract of a stressed host: the role of epithelia cell contact and molecules of the Quorum Sensing Signaling System. *Ann. Surg.* **238**:754-64.

Chapter 5

***In vitro* analysis of Bsh enzyme protection against enteric conditions by whey protein-gum arabic microencapsulation.**



J. M. Lambert
F. Weinbreck
M. Kleerebezem

5. *In vitro* analysis of Bsh enzyme protection against enteric conditions by whey protein-gum arabic microencapsulation

The interest in efficient intestinal delivery of health-promoting substances is increasing. However, the delivery of vulnerable substances such as enzymes requires specific attention. The transit through the stomach, where the pH is very low, can be detrimental to the enzymatic activity of the protein to be delivered. Here we describe the microencapsulation of the model enzyme bile salt hydrolase (Bsh) using whey protein-gum arabic microencapsulates for food-grade and targeted enzyme delivery in the proximal region of the small intestine. Furthermore, the efficacy of enteric coating-microencapsulates for site-specific enzyme delivery was compared *in vitro* with living *Lactobacillus plantarum* WCFS1 bacteria that endogenously produce the Bsh enzyme. Microencapsulates allowed highly effective protection of the enzyme under gastric conditions. Moreover, Bsh release under intestinal conditions appeared to be very efficient, although in the presence of pancreatin, the Bsh activity decreased in time due to proteolytic degradation. In comparison, *L. plantarum* appeared to be capable to withstand gastric conditions as well as pancreatin challenge. Delivery using encapsulates and live bacteria each have different (dis)advantages that are discussed. In conclusion, live bacteria and food-grade microencapsulates provide alternatives for dedicated enteric delivery of specific enzymes and the choice of enzyme to be delivered may determine which mode of delivery is most suitable.

INTRODUCTION

Microencapsulation technology can be used to protect substances from the environment by entrapment into another material. This technology has been used in the food and pharmaceutical industry for controlled release, enhancement of stability, and taste masking in applications such as drugs, vitamins and minerals (for review, see (7) and (8)). Nowadays, the food industry is increasingly focusing on the incorporation of components that provide a health benefit to the customer in food products. The most common and non-invasive mode of administration of health-promoting substances is via the oral route. Therefore, the availability of systems for delivery of specific components into the intestinal tract is of importance. The intestinal delivery of enzymes deserves special attention, since only the slightest change in conformation of a protein can be detrimental to its enzymatic properties. However, the intestinal delivery of proteins or enzymes is severely hampered by denaturation and degradation of the protein in the highly acidic environment in the stomach.

Several methods have been proposed to protect therapeutic proteins during passage of the stomach, such as delivery using viable bacteria (10), and alginate-chitosan encapsulation (3, 20). However, not all encapsulation techniques can be used, as some require the use of high temperatures or organic solvents during the manufacturing process that in many cases prove to be detrimental to the enzyme. A promising system for enzyme encapsulation for intestinal delivery is the use of whey protein-gum arabic encapsulates using activated whey proteins (24), (6). Prior to the encapsulation procedure, activation of the whey proteins with a heat treatment leads to natural cross-linking via disulfide bridges. At the low pH conditions encountered during gastric transit, the activated whey proteins layer remains insoluble. Furthermore, the whey protein and gum arabic layers form an electrostatic complex below the iso-electric point of the protein (i.e. 5.2 for native whey proteins), leading to a tight network protecting the encapsulated enzyme at low pH. Subsequently, at the neutral pH of the duodenum, the coacervate network dissolves and the water-insoluble network of activated whey proteins slowly releases the encapsulated enzyme, with the release profile depending on the extent of cross-linking of the whey protein layer and on the presence of proteases in the environment. Furthermore, activated whey protein-gum arabic encapsulates are label-friendly and food-grade, and are not associated with health and religion-derived dietary issues, which can not be claimed for the more commonly used gelatin-gum arabic coacervates (for examples, see (4, 9, 11, 17)).

The delivery of enzymes protected by whey protein-gum arabic microencapsulates in the proximal small intestine was studied *in vitro*, using the model enzyme bile salt hydrolase (Bsh). Bsh is produced by the commensal bacterial inhabitants of the intestinal tract (2), and deconjugates the amino acid moiety of the bile salts that are produced in the liver and secreted into the proximal small intestine in vertebrates. Thereby, Bsh is an attractive model enzyme to study delivery in the proximal small intestinal tract, since its effective and active delivery would immediately affect the conjugation state of bile acids in this region of the small intestine. Moreover, the delivery efficacy of Bsh by these microencapsulates was compared with delivery by the living model bacterium *L. plantarum* WCFS1 (14), which is a natural Bsh-producing strain (16).

MATERIALS AND METHODS

Chemicals and enzymes. Bsh enzyme of *Clostridium perfringens* (see (1) and (13) for enzymatic properties), pepsin (43 U/mg solid), pancreatin (8x USP) were purchased from Sigma, St. Louis, USA. Whey protein isolate (Bipro) was purchased from Davisco, Eden Prairie, USA and gum arabic (IRX 40693) from Colloïdes Naturels International (Rouen, France).

Preparation of Bsh-whey protein-gum arabic encapsulates. Stomach-resistant Bsh-encapsulates were prepared using a fluidized bed coater (FB-1, Glatt, Binzen, Germany) consisting of a core of inert 350 to 500 μm microcrystalline cellulose spheres (Cellets[®] 350, Syntapharm, Mülheim an der Ruhr, Germany) on which Bsh, whey protein and gum arabic were sprayed (Figure 1A). First, 60 g of a mixture of 7500 U (56 mg) powdered Bsh and 8% (w/v) heat denatured whey protein (pre-heated at 90°C at pH7 for 30 minutes) was coated onto 300 g of Cellets[®] at a product temperature of maximally 42°C. Subsequently, a coating of 30 g of 20% (w/v) gum arabic (pH 7) (Colloïdes Naturels International, Rouen, France) was added at a product temperature of maximally 42°C, resulting in the end-product (Figure 1B).

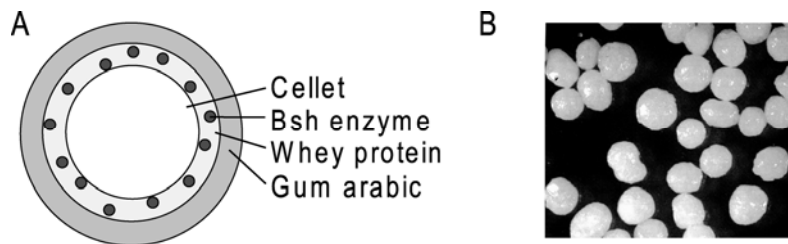


Figure 1. (A) Schematic representation of the Bsh-whey protein-gum arabic encapsulate. (B) Stereoscopic image of the Bsh-whey protein-gum arabic encapsulate (40x magnification).

Simulated gastro-intestinal conditions. Gastric conditions were simulated by the incubation of 0.5 g of microencapsulate, 1 U of free Bsh enzyme, or the Bsh-producing strain *L. plantarum* WCFS1 (14) (for preparation, see below) in 1 ml simulated gastric fluid (SGF) pH 4.5 (5 mM acetate buffer pH 4.5, ionic strength 70 mM) or SGF pH 2 (5mM phosphate buffer pH 2, ionic strength 70 mM) with or without 0.07 g/l pepsin (adapted from (15) and K. Venema, personal communication) for 15 minutes at 37°C. Subsequently, the samples were transferred to intestinal conditions by the addition of 1 ml of pre-warmed simulated intestinal fluid (SIF; 50 mM phosphate buffer pH 7, ionic strength 111 mM (25)) with or without 1.25 g/l pancreatin at 37°C. When no gastric incubation was performed, intestinal conditions were simulated by incubation in a mixture of 1 ml of SGF pH 4.5 and 1 ml of SIF (which is a stronger buffer than SGF) with or without 1.25 g/l pancreatin at 37°C.

***L. plantarum* preparations.** For *L. plantarum* WCFS1 Bsh activity assays, an overnight culture grown in MRS broth (Difco, West Molesey, United Kingdom) at 37°C without aeration was inoculated 1:20 in fresh MRS broth and grown to an OD₆₀₀ of 3. Cells were harvested by centrifugation for 10 minutes at 4500 rpm at room temperature (Megafuge 1.0R, Heraeus, Hanau, Germany) and resuspended in the appropriate SGF-buffer at a final OD₆₀₀ of 50. Of this preparation, 1 ml was used for further assays. When no gastric incubation was performed, a mixture of equal amounts of SGF pH 4.5 and SIF was used for

resuspension of *L. plantarum* cells, and 2 ml of this preparation was used for further assays.

Bsh activity assays. To determine the BSH activity level of Bsh-microencapsulates, 20 μ l of 0.1 M of the bile salt glycocholic acid (GC) was added immediately after transfer to intestinal conditions. At regular time intervals, 40 μ l of sample was transferred to 120 μ l 70% (v/v) acetonitrile to stop the Bsh reaction and to precipitate proteins. HPLC analysis of GC deconjugation was carried out as described below.

Influence of pancreatin on Bsh activity. The influence of pancreatin on the Bsh activity of microencapsulates, free enzyme and *L. plantarum* WCFS1 was started either immediately or after regular time points after transfer to simulated intestinal conditions by addition of 20 μ l of 0.1 M GC. After 30 minutes of incubation with GC at 37°C, 40 μ l of sample was transferred to 120 μ l 70% (v/v) acetonitrile to stop the Bsh reaction and to precipitate proteins. HPLC analysis of GC deconjugation was carried out as described below.

Release properties of Bsh-encapsulate. The release of Bsh from the Bsh microencapsulates was studied by comparison of the input of enzymatic units of Bsh activity at encapsulate assay conditions and the output of activity during simulated intestinal conditions. For this, 0.5 g of Bsh encapsulate and 1U of free Bsh enzyme (which was used as a reference), respectively, were incubated in 2 ml of pre-warmed SIF for 90 minutes at 37°C. Subsequently, 20 μ l of 0.1 M GC was added. At several time points during incubation, 40 μ l of sample was transferred to 120 μ l 70% (v/v) acetonitrile to stop the Bsh activity and to precipitate proteins. HPLC analysis of GC deconjugation was carried out as described below.

HPLC analysis of GC deconjugation. Prior to HPLC analysis, samples obtained during GC deconjugation assays were cleared of proteins and encapsulates by repeated centrifugation at 13200 rpm for 5 minutes at room temperature (Eppendorf AG, Hamburg, Germany). Subsequently, the supernatant was diluted 3.5-fold in double distilled water. GC deconjugation was determined by HPLC as described previously (5). Separations were carried out with a reversed-phase resin-based column (PLRP-S, 5 μ m, 300 Å, 250 x 4.6 mm I. D., Polymer laboratories, Shropshire, UK) and matching pre-column, using an acetonitrile gradient. Bile salts were detected using a pulsed amperometric detector (EG&G Princeton applied research, Princeton, NJ), equipped with a gold working electrode and a reference electrode (Ag/AgCl). Chromatograms were analyzed and integrated using the Chromeleon program (Dionex, Sunnyvale, USA) and the Bsh activity level was calculated on basis of the disappearance of GC.

Microencapsulate imaging techniques. For stereoscopic imaging, Bsh microencapsulates were magnified 40 times using a Leica MZ16 stereoscope; images were captured using Leica IM imaging software (Leica, Rijswijk, the Netherlands). For microscopic imaging, microencapsulates were treated with

SGF with or without pepsin or left untreated. Subsequently, at regular time intervals after transfer to intestinal conditions, some microencapsulates were placed onto a glass slide, immersed in a small amount of SIF, and magnified 312,5 times using a Leica Dialux 20 microscope; images were captured using a COHU High Performance CCD camera and Leica QFluoro imaging software (Leica, Rijswijk, the Netherlands).

RESULTS AND DISCUSSION

Bsh-microencapsulation. The Bsh enzyme was chosen as a model enzyme to study the enteric delivery properties of whey protein-gum arabic microencapsulates, using *in vitro* conditions mimicking physicochemical conditions encountered during gastric transit and upon proximal small intestinal entry. This food-grade delivery concept aims to protect the enzyme during gastric conditions, while allowing dedicated release into the lumen of the proximal small intestine. Bsh-microencapsulates were manufactured using the fluidized bed coating technique, applying a coat of a mixture of Bsh enzyme and heat denatured whey protein onto microcrystalline cellulose cellets. The heat-treatment caused the whey proteins to form a coating linked by disulfide bonds, lowering the total porosity of the whey protein layer and providing lowered solubility of the coating in water (6), (21). Subsequently, a coat of gum arabic was added (Figure 1), enabling the reversible formation of a protein-gum arabic coacervate (24) at low pH.

Microencapsulate protection against gastric conditions. The influence of various gastro-intestinal treatments on the physical appearance of the Bsh microencapsulates was monitored by microscopical analysis (Figure 2A). The physical appearance of the microencapsulates did not appear to be altered under gastric conditions (SGF) either in the presence or absence of gastric pepsin (Figure 2A). Gastric conditions were applied by incubation of the Bsh-encapsulates in SGF at pH 2 or pH 4.5, respectively, representing either the fasting or the fed state in humans (12, 15). In agreement with the physical appearance of the microencapsulates, the bile salt hydrolase activity of the microencapsulates under simulated intestinal conditions in absence of pancreatin appeared to be independent of the type of gastric incubation condition used (i.e., incubation at pH 2 or pH 4.5 and in presence or absence of pepsin, or no gastric incubation), with Bsh activity ranging from 10 to 12 nmol/min (Figure 3). These findings indicate a strong protective effect of the whey protein-gum arabic coacervate against stomach conditions.

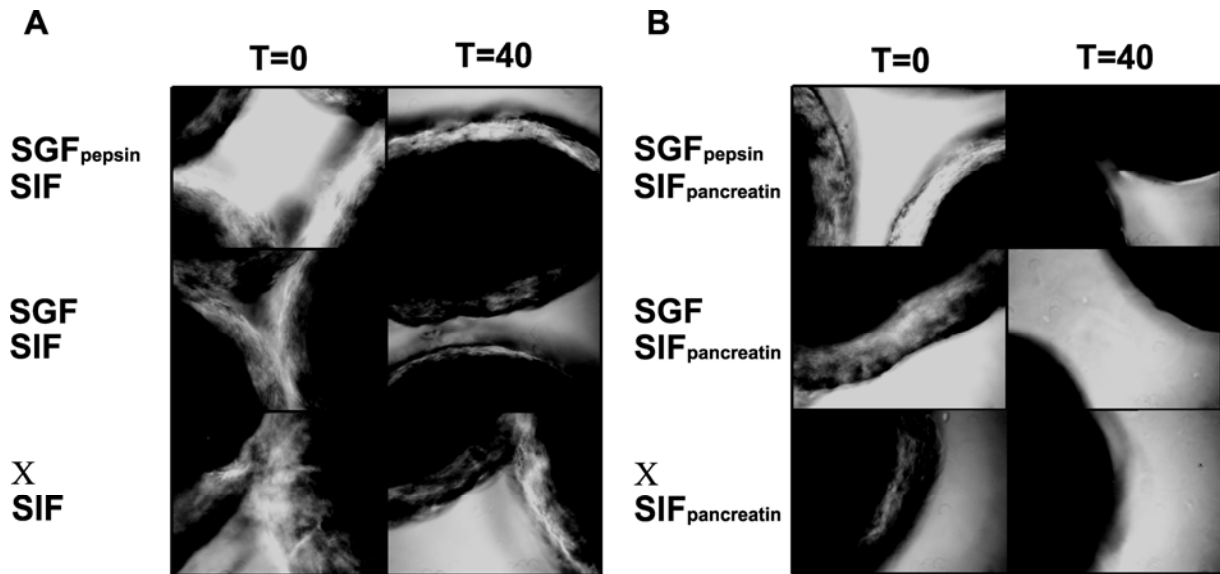


Figure 2. Microscopic images (312.5 x magnification) of the Bsh-whey protein-gum arabic coat of the Bsh-encapsulates. When appropriate, Bsh-encapsulates were pre-treated in SGF with or without pepsin, and images were made at the start of the SIF-incubation (T=0) and after 40 minutes of SIF incubation (T=40). The Bsh-whey protein-gum arabic coating on the cellets was visible as a transparent layer. Shown is the intestinal incubation of the microencapsulates in the absence (panel A) and presence (panel B) of pancreatin. Independent of the gastric pre-treatment condition, the encapsulate coat was dissolved only in the presence of pancreatin during the SIF-treatment. SGF_{pepsin}, SGF containing pepsin; SIF_{pancreatin}, SIF containing pancreatin; X, no pre-treatment.

Intestinal Bsh activity from microencapsulates. In the absence of pancreatin during intestinal conditions, the amount of GC deconjugated increased linearly in time (Figure 3), indicating a constant rate of GC deconjugation. No lag-phase in bile salt hydrolase activity was observed; thus, it can be concluded that bile salt hydrolase activity from the microencapsulates was available immediately following transfer to intestinal conditions, as desired. Notably, these findings were not reflected macroscopically (Figure 2A). In the absence of pancreatin, the encapsulate coating was not dissolved during intestinal conditions. Upon entrance of the encapsulates into the intestine, the increase in pH led to the de-complexation of the gum arabic and whey protein layer complex. In addition, at the pH of the intestine, the activated whey protein provided a less tight network than at gastric pH, enabling the bile salt substrate to permeate into the microencapsulate layer, without total release of the relatively large Bsh enzyme from the microencapsulate being necessary. Apparently, intestinal delivery of Bsh activity from microencapsulates is highly efficient, regardless of physical release from the microencapsulate. Furthermore, Bsh-activity from the encapsulates under intestinal conditions in absence of pancreatin was approximately 10 % of the theoretical input. Since intestinal delivery of Bsh from the microencapsulates was highly efficient, the loss of activity was very likely due to processing conditions during the fluid bed

coating procedure. The relative instability of the Bsh enzyme in solution (data not shown) may be of importance here, and activity retainment could probably be improved by reduction of processing time. In addition, the process temperature could be decreased to limit activity loss by heat-damage, but as a consequence, preparation time would be increased. Alternatively, instead of the spraying of the Bsh enzyme onto cellets, where the Bsh enzyme remains in solution for a relatively long time, the Bsh enzyme could be incorporated into core particles prepared by an encapsulation method that involves reduction of the amount of water in the particles (such as extrusion). Subsequently, the encapsulate core containing the stabilized Bsh enzyme would be coated with a protective layer of activated whey protein and gum arabic.

Furthermore, after one month of dry storage at 4 °C, Bsh activity in the microencapsulates was fully retained (data not shown), illustrating the excellent shelf life properties of these encapsulates.

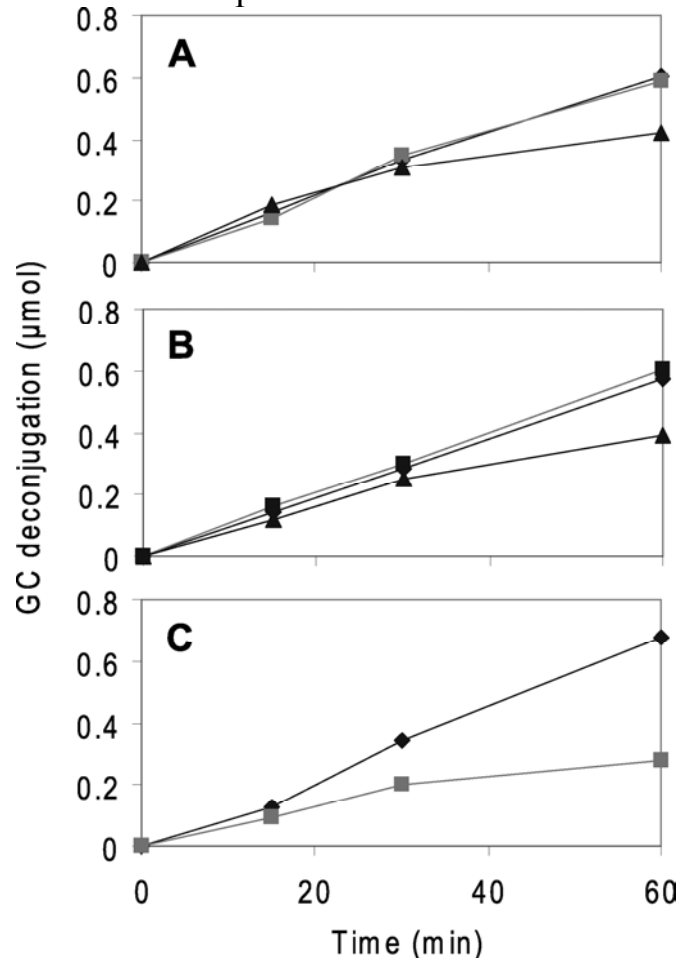


Figure 3. (A). GC deconjugation by 0.5 g Bsh-encapsulate in time following treatment in SGF with or without pepsin at pH 2. ◆ treatment with SGF, activity in SIF; ■ treatment with SGF with pepsin, activity in SIF; ▲ treatment with SGF, activity in SIF with pancreatin. (B). GC deconjugation by 0.5 g Bsh-encapsulate in time following treatment in SGF with or without pepsin at pH 4.5. ◆ treatment with SGF, activity in SIF; ■ treatment with SGF with pepsin, activity in SIF; ▲ treatment with SGF, activity in SIF with pancreatin. (C). GC deconjugation by 0.5 g Bsh-encapsulate in time without prior treatment in SGF. ◆ activity in SIF; ■ activity in SIF with pancreatin.

Influence of pancreatin on intestinal Bsh activity. The presence of pancreatin during intestinal incubation had a major effect on the physical appearance of the Bsh encapsulates, as determined by microscopical analysis (Figure 2A and 2B). The digestive action of pancreatin totally dissolved the Bsh-whey protein-gum arabic layer of the microencapsulates, whereas in absence of pancreatin, this layer remained intact. Thus, pancreatin may enhance release of enzymes from the microencapsulate layer.

In contrast, in the presence of pancreatin during intestinal conditions, the Bsh activity from microencapsulates was found to decrease parabolically (Figure 3). The pancreatin-dependent decrease of Bsh activity in time of the microencapsulates probably indicated that the Bsh enzyme was degraded by proteolytic enzymes present in pancreatin. Thus, pancreatin may have a dual action in delivery of enzymes in the small intestine. Pancreatin may enhance release of enzymes from the microencapsulate layer, but it will also degrade the released enzyme. This is in agreement with the US Pharmacopeia (22), where the use of pancreatin was found to be of importance for *in vitro* oral drug release assays.

Notably, the Bsh activity from the microencapsulates in presence of pancreatin did not exceed the activity in absence of pancreatin (Figure 3). Thus, in our experiments, total release of the Bsh-whey protein-gum arabic layer as observed during microscopical analysis (Figure 2B) appeared non-beneficial for obtaining higher Bsh activity during intestinal conditions in the presence of pancreatin. This is in agreement with the finding that Bsh activity is available immediately after transfer to intestinal conditions in absence of pancreatin and thus excluding physical release of the microencapsulate layer, as described above.

Furthermore, the formation of a coacervate layer during gastric incubation may provide protection against the proteolytic activity of pancreatin while still allowing for Bsh activity upon entrance into intestinal conditions. This suggestion is supported by the fact that a slightly larger decrease in bile salt deconjugation was found in response to the presence of pancreatin during intestinal conditions with no gastric pre-incubation as compared to gastric pre-incubation at pH 2 or pH 4.5 (Figure 3) (0.28 μmol , 0.42 μmol , and 0.39 μmol GC hydrolysed in 60 minutes, respectively). The protective effect of the formation of a whey protein-gum arabic coacervate layer on Bsh activity against pancreatin pressure was also found in a pancreatin resistance assay, where Bsh activity was monitored after various pancreatin incubation times with gastric pre-incubation at pH 2 or pH 4.5 in the presence of pepsin (Figure 4A). Here, gastric pre-incubation at pH 2 appeared to confer a slight advantage over pre-incubation at pH 4.5. However, more investigation is needed to establish this effect further and eventually unravel the underlying mechanism.

Gastro-intestinal incubation of free Bsh (Figure 4B) clearly shows that only a small amount of Bsh activity was left after gastric incubation in the presence of

pepsin (at assay starting time 0), with a more severe reduction of activity at gastric incubation at pH 2 compared to pH 4.5.

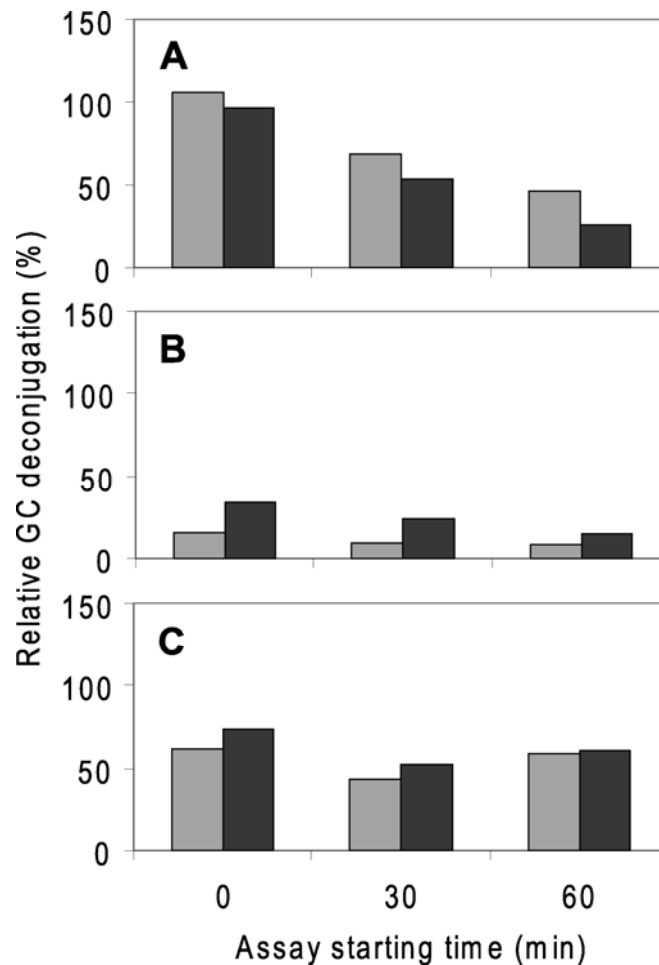


Figure 4. GC deconjugation in response to pancreatin pressure during simulated intestinal conditions. Following treatment with SGF containing pepsin for 15 minutes, the GC deconjugation levels of (A) Bsh-encapsulate, (B) free Bsh enzyme, and (C) *L. plantarum* WCFS1, respectively, were determined in SIF containing pancreatin. To determine the effect of various pancreatin treatment times, the GC deconjugation assay was started at various time points after transfer to simulated SIF containing pancreatin. GC deconjugation levels are shown relative to the GC deconjugation in SIF without prior SGF-treatment, which was taken as a reference condition (that is, 20 nmol/g/min for encapsulate, 1 nmol/ml/min for *L. plantarum* culture at an OD₆₀₀ of 3, and 40 nmol/g/min for free Bsh, respectively). White bars represent pre-treatment with SGF pH 2; black bars represent pre-treatment with SGF pH 4.5.

Clearly, the enzyme is broken down further by pancreatin activity. As expected, the use of free enzyme is not recommended for delivery of Bsh activity in the proximal small intestine.

However, an alternative to encapsulation of enzyme is the use of viable bacteria for delivery of enzymes in the small intestine (10). In order to evaluate this alternative, the bacterial strain *L. plantarum* WCFS1 that natively produces the Bsh-enzyme intracellularly (16) and that is capable of surviving the gastro-

intestinal tract (23) was used as a model. Notably, the native amount of Bsh activity produced per ml of *L. plantarum* WCFS1 culture at an OD₆₀₀ of 3 was 20 times lower as compared to activity per gram of encapsulate. Importantly, *L. plantarum* WCFS1 appeared to be highly pancreatin-resistant in time (Figure 4C). This characteristic may provide a particular advantage over encapsulate delivery. For *L. plantarum*, gastric incubation at pH 2 initially appeared to provide a minor negative effect on Bsh-activity as compared to pH 4.5, with gastric incubation decreasing Bsh-activity by approximately 50 %. However, after 60 minutes of incubation, *L. plantarum* appeared to have recuperated from gastric conditions, showing no difference in Bsh-activity for both gastric incubation treatments (Figure 4C). Thus, although bacterial delivery may be limited quantitatively, it appears to be a more stable means of delivery under the conditions of the small intestine as compared to delivery using encapsulates.

CONCLUSION

Whey protein-gum arabic encapsulation is an excellent tool to protect enzymes during transit through gastric conditions and release enzyme activity in the proximal small intestine. However, following transfer to intestinal conditions, the enzyme is released by and exposed to the activity of pancreatin, and other proteolytic activities *in vivo*. Since *L. plantarum* WCFS1 is capable of surviving gastric conditions and is able to withstand pancreatin pressure, it may be a good alternative for intestinal delivery of enzymes.

However, several issues need to be considered. For example, the ease of handling of encapsulates in terms of storage and shelf life may be preferred as compared to viable bacteria. Furthermore, the exact site of delivery using live bacteria is less well predictable compared to microencapsulates. In addition, the expression level of enzymes is limited by the constraints on protein production in live bacteria. In microencapsulates, the amount of enzyme that can be incorporated is virtually unlimited due to the usually high activity-to-weight ratio of enzymes. However, especially in industrial systems, the cost of purified enzymes to be used in microencapsulates may be an important issue; use of crude enzyme extracts could in many cases provide cost reduction.

Furthermore, the use of genetically modified organisms (GMOs) for both production and delivery of enzymes in the intestinal tract could offer several advantages. The expression level of enzymes could be improved using over-expression systems such as the food-grade NICE system (18), in combination with an appropriate food-grade selection marker (19), when desired. In addition, enzyme sequences could be optimized by strategies such as site-directed mutagenesis to improve stability during microencapsulation procedures, protease resistance, or activity level under intestinal conditions. GMO strategies may also be preferred for the delivery of human enzymes. Furthermore, the perception of oral intake of viable bacteria may be perceived differently as

compared to encapsulates for use in the food industry. However, application of GMO derived products in food, even when genetic modifications are performed using food-grade cloning strategies, are still subject to debate. Moreover, expression of some enzymes may only be achievable using genes obtained from non-food-grade hosts.

Thus, the system to be used for intestinal delivery of enzymes largely depends of the enzyme to be delivered.

ACKNOWLEDGEMENTS

The authors would like to thank Roelof van der Meer for his valuable contribution to this manuscript.

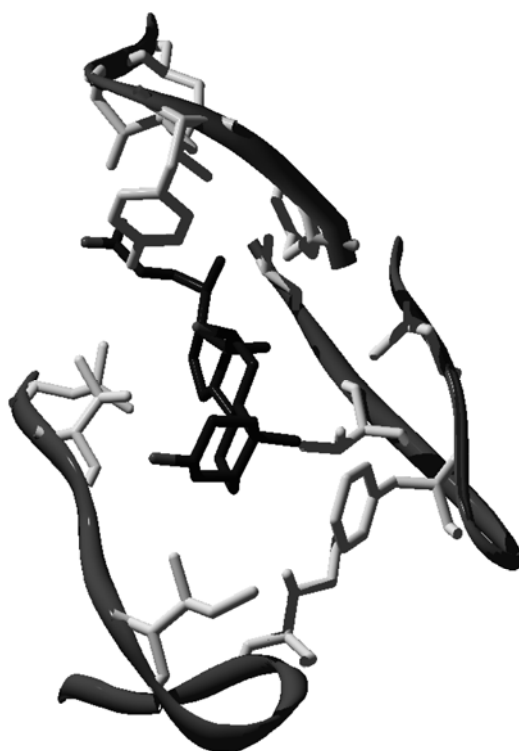
REFERENCES

1. **Batta, A. K., G. Salen, and S. Shefer.** 1984. Substrate specificity of cholyglycine hydrolase for the hydrolysis of bile acid conjugates, p. 15035-15039, vol. 259.
2. **Begley, M., C. Hill, and C. G. Gahan.** 2006. Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol.* **72**:1729-38.
3. **Bhopatkar, D., A. K. Anal, and W. F. Stevens.** 2005. Iontropic alginate beads for controlled intestinal protein delivery: effect of chitosan and barium counter-ions on entrapment and release. *J. Microencapsul.* **22**:91-100.
4. **Chang, C. P., T. K. Leung, S. M. Lin, and C. C. Hsu.** 2006. Release properties on gelatin-gum arabic microcapsules containing camphor oil with added polystyrene. *Colloids Surf. B Biointerfaces* **50**:136-40.
5. **Dekker, R., Meer, R. van der, Olieman, C.** 1991. Sensitive pulsed amperometric detection of free and conjugated bile acids in combination with gradient reversed-phase HPLC. *Chromatographia* **31**:549-553.
6. **Floris, R., Bodnár, I, Weinbreck, F, Alting A. C.** 2007. Dynamic rearrangement of disulfide bridges influences solubility of whey protein coatings. Accepted for publication in *Int. Dairy J.*
7. **Gibbs, B. F., S. Kermasha, I. Alli, and C. N. Mulligan.** 1999. Encapsulation in the food industry: a review. *Int. J. Food Sci. Nutr.* **50**:213-24.
8. **Gouin, S.** 2004. Micro-encapsulation: industrial appraisal of existing technologies and trends. *Trends Food Sci. Tech.* **15**:18.
9. **Huang, Y. I., Y. H. Cheng, C. C. Yu, T. R. Tsai, and T. M. Cham.** 2007. Microencapsulation of extract containing shikonin using gelatin-acacia coacervation method: A formaldehyde-free approach. *Colloids Surf. B Biointerfaces* **58**:290-7.
10. **Huyghebaert, N., A. Vermeire, P. Rottiers, E. Remaut, and J. P. Remon.** 2005. Development of an enteric-coated, layered multi-particulate formulation for ileal delivery of viable recombinant *Lactococcus lactis*. *Eur. J. Pharm. Biopharm.* **61**:134-41.
11. **Junyaprasert, V. B., A. Mitrevej, N. Sinchaipanid, P. Boonme, and D. E. Wurster.** 2001. Effect of process variables on the microencapsulation of vitamin A palmitate by gelatin-acacia coacervation. *Drug Dev. Ind. Pharm.* **27**:561-6.
12. **Kararli, T. T.** 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm. Drug Dispos.* **16**:351-80.

13. Kirby, L. C., Klein, R. A., Coleman, J. P. 1995. Continuous spectrophotometric assay of conjugated bile acid hydrolase. *Lipids* **30**:863-867.
14. Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen. 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. USA* **100**:1990-5.
15. Krul, C., A. Luiten-Schuite, R. Baandagge, H. Verhagen, G. Mohn, V. Feron, and R. Havenaar. 2000. Application of a dynamic in vitro gastrointestinal tract model to study the availability of food mutagens, using heterocyclic aromatic amines as model compounds. *Food Chem. Toxicol.* **38**:783-92.
16. Lambert, J. M., R. S. Bongers, and M. Kleerebezem. 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **73**:1126-35.
17. Lamprecht, A., U. Schafer, and C. M. Lehr. 2001. Influences of process parameters on preparation of microparticle used as a carrier system for omega - 3 unsaturated fatty acid ethyl esters used in supplementary nutrition. *J. Microencapsul.* **18**:347-57.
18. Mierau, I., and M. Kleerebezem. 2005. 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Appl. Microbiol. Biotechnol.* **68**:705-17.
19. Platteeuw, C., I. van Alen-Boerrigter, S. van Schalkwijk, and W. M. de Vos. 1996. Food-grade cloning and expression system for *Lactococcus lactis*. *Appl. Environ. Microbiol.* **62**:1008-13.
20. Ramadas, M., W. Paul, K. J. Dileep, Y. Anitha, and C. P. Sharma. 2000. Lipoinsulin encapsulated alginate-chitosan capsules: intestinal delivery in diabetic rats. *J. Microencapsul.* **17**:405-11.
21. Rosenberg, M., and S. J. Lee. 2004. Calcium-alginate coated, whey protein-based microspheres: preparation, some properties and opportunities. *J. Microencapsul.* **21**:263-81.
22. United States Pharmacopeial Convention, I. 2000. U. S. Pharmacopoeia XXIV/National Formulary XIX 2000, Rockville, MD 7.
23. Vesa, T., P. Pochart, and P. Marteau. 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. *Aliment. Pharmacol. Ther.* **14**:823-8.
24. Weinbreck, F., M. Minor, and C. G. de Kruif. 2004. Microencapsulation of oils using whey protein/gum Arabic coacervates. *J. Microencapsul.* **21**:667-79.
25. Yoo, S. H., Y. B. Song, P. S. Chang, and H. G. Lee. 2006. Microencapsulation of alpha-tocopherol using sodium alginate and its controlled release properties. *Int. J. Biol. Macromol.* **38**:25-30.

Chapter 6.

***In vivo* modulation of host response by intestinal delivery of bile salt hydrolase activity.**



J. M. Lambert
S. J. M. ten Bruggencate
R. van der Meer
M. Kleerebezem

6. *In vivo* modulation of host response by intestinal delivery of bile salt hydrolase activity

In the liver of mammals, bile salts are synthesised from cholesterol and conjugated to either taurine or glycine. Following release into the intestine, conjugated bile salts can be deconjugated by members of the endogenous microbiota that produce an enzyme called bile salt hydrolase. Bile salt deconjugation has been suggested to play an important role in the physiology of the intestine. Furthermore, a commonly used criterion for the selection of candidate probiotic strains is their capability to hydrolyse bile salts. However, the administration of large amounts of bile salt-hydrolysing bacteria could potentially result in a shift in intestinal bile salt composition that might be associated with an undesirable impact on intestinal physiology. To investigate the influence of small intestinal delivery of bile salt hydrolase on the host, the lactic acid bacterium *Lactobacillus plantarum* WCFS1 was used as a model for the administration of bile salt-hydrolysing probiotic strains. In addition, Bsh enzyme was delivered to the small intestine using protein-gum arabic encapsulates. The influence of delivery of Bsh enzyme to the small intestinal tract on the host was investigated by determination of the *in vivo* bile salt composition and mucin excretion levels. However, no effect of delivery of Bsh on the intestinal bile salt composition or mucin excretion was detected. These results indicate that the physiological relevance and magnitude of bile salt hydrolase activity of probiotics in the small intestine is limited.

INTRODUCTION

Primary bile salts are synthesised from cholesterol in the liver of mammals, where they are conjugated to either taurine or glycine. The type of amino acid conjugated to the steroid nucleus can be influenced by dietary components, such as pectin and cholesterol (13-15), and the availability of taurine or glycine in the diet (11). Since taurine is rare in plants but widely available in meat and seafood, the bile salts of herbivores are mostly conjugated to glycine, whereas the bile salts of carnivores are mostly conjugated to taurine (1). Analogously, in omnivores, bile salts are conjugated to both glycine and taurine. Furthermore, under normal physiological conditions, one or more hydroxyl groups can be added to the steroid nucleus of bile salts, rendering the molecule less hydrophobic (1). Thus, conjugated bile salts are amphipathic molecules that, following secretion into the duodenum, play an important role in the adsorption and digestion of lipids and lipid-soluble vitamins. The majority of the bile salts recirculate to the liver by the portal blood by mostly active transport in the terminal ileum.

In the intestine, conjugated bile salts can be deconjugated by members of the endogenous microbiota that produce an enzyme called bile salt hydrolase (Bsh; EC3.5.1.24; for review, see (3)), releasing the glycine or taurine moiety from the bile salt. Previously, especially lactobacilli were reported to be responsible for the majority of bile salt hydrolase activity in the small intestine (8, 29, 30); e.g., in lactobacillus-free mice, the Bsh activity in the ileal contents was found to be reduced by almost 90 % when compared to lactobacillus-reconstituted mice (29).

Bile salt deconjugation plays an important role in the physiology of the intestine, since it is the first step in further bile salt transformations by intestinal bacteria, such as dehydroxylation reactions, leading to secondary bile salts that are associated with various intestinal diseases such as the formation of gallstones and colon cancer (19, 25). In addition, *in vitro* and *ex vivo* studies have suggested that bile salt deconjugation plays a role in stimulation of mucin production and excretion into the intestinal lumen (20), which could influence intestinal transit (26). Mucin consists of a network of glycoproteins that acts as a protective layer for the epithelial surfaces. Therefore, an increase in mucin production by epithelial cells may be considered as a host response to irritation caused by the formation of deconjugated bile salts by bacterial bile salt hydrolases.

Bile salt hydrolases have been found in a wide variety of mainly Gram-positive species that usually are inhabitants of the intestinal tract. The ability to hydrolyse bile salts has been suggested to be of importance to the survival and persistence of bacterial strains in the gastro-intestinal tract (for review, see (3)). Consequently, a commonly used criterion for the selection of candidate probiotic strains is their capability to hydrolyse bile salts. However, the administration of large amounts of bile salt-hydrolysing bacteria could potentially result in a shift in intestinal bile salt composition. This might be associated with an undesirable impact on intestinal physiology. Therefore, the influence of the delivery of bile salt hydrolase activity to the intestine on host response deserves attention.

In order to deliver bile salt hydrolase activity *in vivo*, several delivery vehicles can be considered, such as viable bacteria (12) and/or various encapsulation methods (5, 10, 23, 33). Here we investigated the influence of the delivery of bile salt hydrolase activity to the small intestine of rats by either viable bacteria or encapsulates. To approximate the human situation as much as possible, the diet fed to the rats had a low calcium and high fat content, simulating the composition of a Western human diet (31). As a model for the administration of bile salt-hydrolysing probiotic strains, the lactic acid bacterium *Lactobacillus plantarum* WCFS1 (18) was used, which is a natural bile salt hydrolase producer (21). Notably, *L. plantarum* WCFS1 has been shown to survive in the gastro-intestinal tract for up to ten days (22, 32). For delivery of Bsh enzyme using microencapsulates, Bsh-containing whey protein-gum arabic

microencapsulates were used, of which the characteristics were previously found to be excellent for protection against stomach conditions and effective delivery in the small intestine (Chapter 5). The influence of delivery of Bsh enzyme to the small intestinal tract on the host was investigated by determination of the *in vivo* bile salt composition and mucin excretion levels.

MATERIALS AND METHODS

Animals and diets. The experimental protocols for two separate animal studies (i.e., *L. plantarum* and Bsh encapsulate intake, respectively) were approved by the animal welfare commission of Wageningen University, Wageningen, The Netherlands, and were conducted according to Dutch regulations and law. Two groups of $n = 9$ (for *L. plantarum* intake) or $n = 11$ (for Bsh encapsulate intake) per group male Fischer F344 rats (Charles River, Maastricht, The Netherlands) were housed individually in grid cages with controlled temperature (22 to 24 °C), a relative humidity of 50 to 60 %, and a light/dark cycle with lights on from 6:00 to 18:00h. All rats were fed a Western style humanized purified diet of 200 g/kg acid casein, 278 g/kg corn starch, 172 g/kg glucose, 155 g/kg palm oil containing vitamin A, D, E, and K according to AIN 93 (24), 5 g/kg cholesterol, 40 g/kg corn oil, 10 g/kg vitamin mix and 35 g/kg mineral mix according to AIN 93 (24), 50 g/kg citrus pectin, 50 g/kg cellulose, and 5.16 g/kg CaHPO₄. The animals had free access to demineralised drinking water. Food intake was recorded daily and body weight was recorded every two days.

Oral intake of *L. plantarum* WCFS1. After acclimatisation to the housing and dietary conditions for seven days, the two groups of rats were fed approximately 5×10^{10} colony forming units of either heat-killed or freshly cultured and viable *L. plantarum* WCFS1 (18) (previously adapted to be resistant to 50 µg/ml rifampicin) for eight days (day 1 to 8). For this purpose, an overnight culture of rifampicin-adapted *L. plantarum* WCFS1 grown in MRS broth (Difco, West Molesey, United Kingdom) supplemented with 50 µg/ml rifampicin at 37 °C without aeration was inoculated in fresh MRS broth and grown to an optical density at 600 nm (OD₆₀₀) of 3. When appropriate, cells were killed by heating at 80 °C for 13 minutes, and absence of growth was confirmed by plating of cells on MRS agar (Difco, West Molesey, United Kingdom). Lack of Bsh activity from the heat-killed cells was confirmed by HPLC analysis (see below). Heat-killed and viable cells were harvested by centrifugation for 10 minutes at 4000 rpm at room temperature (Megafuge 1.0R, Heraeus, Hanau, Germany), concentrated ten times in fresh MRS broth to an OD₆₀₀ of 30, and mixed with 2 volumes of UHT-treated custard (Stabilac, Campina, The Netherlands).

Rats were fed 3 g of the freshly prepared custard mixture per day. During the last 24 hours before dissection of the animals, faeces was collected from the

bottom of the grid cages of the animals and frozen at -20 °C for bile salt and mucin analyses (see below). Prior to dissection, fresh faecal samples were collected directly from the anus of the animals and analyzed for viable *L. plantarum* WCFS1 by plating of dilutions of the faeces in saline on MRS agar with and without 50 µg/ml rifampicin and incubation for two days at 37 °C. Five animals belonging to the group of animals fed heat-killed *L. plantarum* WCFS1 rif^R were excluded from analyses due to *L. plantarum* WCFS1 rif^R found in fresh faeces, as determined by plate counting and RAPD analyses (data not shown).

Furthermore, 3 animals of each group (fed viable or heat-killed *L. plantarum* WCFS1 rif^R, respectively) were sedated using isoflurane and bile was collected for 30 minutes by canulation of the bile duct. Bile was stored at 4 °C. Subsequently, all animals were killed by carbon dioxide inhalation. The entire small intestine was removed and dissected into three parts equal in length. The contents of the small intestinal fragments were collected for bile salt and mucin analyses (see below) and kept at -80 °C.

Oral intake of Bsh encapsulates. Stomach-resistant encapsulates designed for delivery into the small intestine were prepared as described earlier (Chapter 5) using the fluidized bed coating technique. Briefly, layers of whey protein with or without (for placebo) Bsh enzyme (Sigma, Zwijndrecht, the Netherlands) and gum arabic (pH 7) (Colloïdes Naturels International, Rouen, France) were sprayed onto a core of inert 350 to 500 µm microcrystalline cellulose spheres (Cellets[®] 350, Syntapharm, Mülheim an der Ruhr, Germany). After acclimatization to the housing and dietary conditions for thirteen days, the two groups of rats were fed 11 g of purified diet per day. Furthermore, rats were acclimatized to the feeding of 2.5 g of placebo encapsulate dispersed in 2.5 g of apple sauce for five days (day 1 to 5). Subsequently, rats were fed either 2.5 g of placebo or 2.5 g of Bsh encapsulate dispersed in 2.5 g of apple sauce for two days (day 6 and 7). On day 7, all animals were killed by carbon dioxide inhalation. The entire small intestine was removed and dissected into three parts equal in length. The contents of the small intestinal fragments were collected for bile salt and mucin analyses (see below) and kept at -80 °C.

Mucin determination. Mucins were extracted from faeces and intestinal contents and quantified fluorimetrically, as described earlier (6). Briefly, faecal and intestinal extracts in phosphate buffered saline were filtrated (Ultrafree MC NMWL, cut off 30000 Da, Millipore, Bedford, USA) by centrifugation for 30 minutes at 3000 g, followed by washing with 100 % methanol. After drying by air, the mucin retentate was solubilised in the original volume of phosphate buffered saline. Total mucin concentration was determined fluorimetrically using addition of 2-cyano-acetamide (7) to the mucin oligosaccharide chains liberated by alkaline β-elimination, with excitation at 336 nm and emission at 383 nm. Recovery was determined by standard addition procedures. Standard solutions of *N*-acetylgalactosamine (Sigma, Zwijndrecht, The Netherlands) were

used to calculate the amount of oligosaccharide side chains liberated from total mucin. Therefore, the amount of mucin is expressed as oligosaccharide equivalents.

HPLC determination of Bsh activity and bile salt composition. Bsh activity of *L. plantarum* WCFS1 rif^R was determined essentially as described earlier for *L. plantarum* WCFS1 (21). Briefly, *L. plantarum* cells were concentrated to an OD₆₀₀ of 50 in MRS. Subsequently, 1 mM of the bile salt glycocholic acid (GC; Sigma, Zwijndrecht, The Netherlands) was added or cells were mixed 9:1 with rat bile (v/v) or intestinal contents (w/v). Conversion of GC and the bile salt composition of faeces and intestinal contents was determined by HPLC as described previously (9). Prior to HPLC analysis, faecal and intestinal samples were cleared by dispersion in 70 % acetonitrile and repeated centrifugation at 13200 rpm for 5 minutes at room temperature (Eppendorf AG, Hamburg, Germany), followed by 3.5-fold dilution in water. Separations were carried out with a reversed-phase resin-based column (PLRP-S, 5 µm, 300 Å, 250 x 4.6 mm I. D., Polymer laboratories, Shropshire, UK) and matching pre-column, using an acetonitrile gradient. Bile salts were detected using a pulsed amperometric detector (EG&G Princeton applied research, Princeton, NJ), equipped with a gold working electrode and a reference electrode (Ag/AgCl). Chromatograms were analyzed and integrated using the Chromeleon program (Dionex, Sunnyvale, USA).

RESULTS AND DISCUSSION

In this work, the influence of bile salt hydrolase delivery in the small intestine of a rat model by *L. plantarum* WCFS1 and whey protein-gum arabic microencapsulates on bile salt composition and mucin excretion was determined. To approximate the human situation as much as possible, the diet fed to the rats had a low calcium and high fat content, simulating the composition of a Western human diet (31). Furthermore, rats naturally produce bile salts conjugated to glycine and taurine in a ratio ranging from 0.15 to 0.35 (13-15), whereas this ratio is approximately 3 in humans (4, 9, 27). In addition, both *L. plantarum* WCFS1 and the encapsulated Bsh enzyme used here have a strong preference for glycine-conjugated over taurine-conjugated bile salts (Chapter 4 and Chapter 5), (2, 17). To mimic human conditions and to maximize the *in vivo* activity of the Bsh enzyme delivered in the intestine, cholesterol and pectin were added to the rat diet, shifting the synthesis of conjugated bile salts in the liver from predominantly taurine- to predominantly glycine-conjugated, resulting in a glycocholic/taurocholic acid ratio of 7±2 (Figure 1A, chromatogram A1), which is in agreement with earlier findings (13-15).

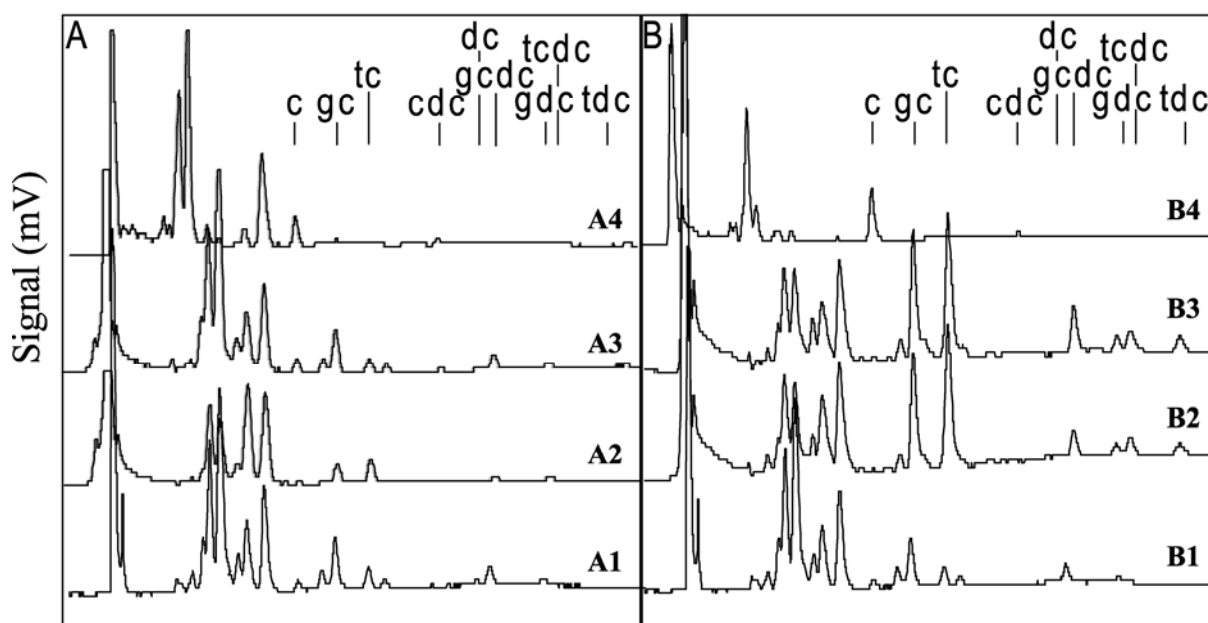


Figure 1. Schematic representation of exemplary HPLC chromatograms of rat bile, intestinal contents, and faeces. Panel A shows rat bile (A1), the contents of the distal part of the small intestine after *in vivo* administration of heat-killed (A2) or viable (A3) *L. plantarum* WCFS1, respectively, and faeces (A4) of control rats is shown. Panel B shows rat bile (B1), and the contents of the distal part of the small intestine after *in vivo* administration of placebo (B2) or Bsh (B3) encapsulates, respectively, and faeces (B4) of control rats is shown. Peak positions of bile salts used as a standard are shown. C, cholic acid; GC, glycocholic acid; TC, taurocholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; GCDc, glycochenodeoxycholic acid; GDC, glycodeoxycholic acid; TCDC, taurodeoxycholic acid; TDC, taurodeoxycholic acid. Total HPLC retention time was 40 minutes.

In most animal models, the presence of a natural microbiota in the intestinal tract would be considered desirable. However, naturally residing lactobacilli have been reported to contribute greatly to the total bile salt hydrolase activity in the small intestine of mice (29, 30), possibly leading to undesirable background bile salt deconjugation levels. However, in our rat model, background bile salt deconjugation levels in the small intestine were very low, as apparent from comparative analysis of the bile salt composition of bile harvested directly from the bile duct and of the small intestinal contents of control rats (Figure 1, chromatogram A1 and A2; B1 and B2). Thereby, this model is highly suitable for the measurement of bile salt deconjugation *in vivo* in the presence of a conventional microbiota. Apparently, bile salt deconjugation by the natural microbiota occurred mostly in the colon, as exemplified by the high bile salt deconjugation levels found in faeces as compared with the small intestinal contents (Figure 2 and Figure 1, chromatogram A2 and A4; B2 and B4).

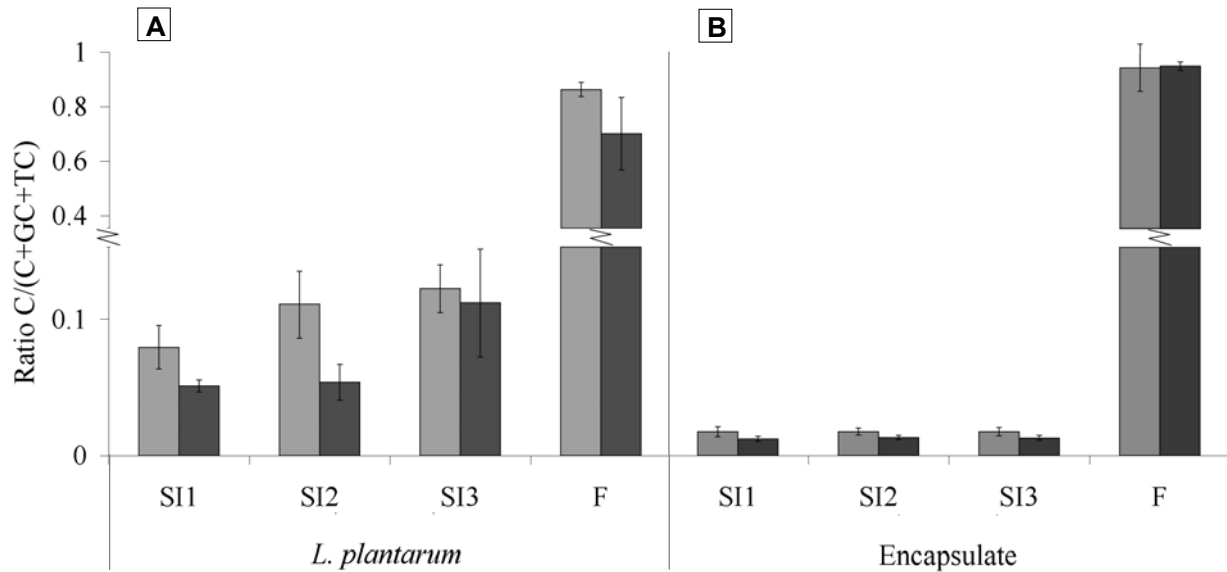


Figure 2. *In vivo* deconjugation of bile salts by *L. plantarum* WCFS1 (panel A), and Bsh encapsulates (panel B), respectively, represented by the ratio of the amount of deconjugated bile salt cholic acid (C) to the total cholic acid pool (cholic acid, and its conjugated forms glyco- and taurocholic acid [GC and TC]) present in the proximal, middle, and distal part of the small intestine (SI1, SI2, and SI3, respectively) and in faeces (F). Light grey bars represent the cholic acid deconjugation ratio in animals fed living *L. plantarum* WCFS1 or Bsh-encapsulate, respectively; dark grey bars represent the cholic acid deconjugation ratio in animals fed heat-killed *L. plantarum* WCFS1 or placebo encapsulate, respectively. Error bars represent the standard error of the mean (SEM) of n=9 or n=4 for viable and heat-killed *L. plantarum*, respectively, and n=11 for Bsh or placebo encapsulate.

Most Bsh enzymes show a preference for glycine-conjugated bile salts (3), and this preference appeared to be reflected in the relative deconjugation of taurocholic and glycocholic acid by the microbiota present in the colon. For example, in the rats fed *L. plantarum* WCFS1, 70% of the conjugated cholic acid that was hydrolyzed in the colon consisted of glycocholic acid, and the remainder consisted of taurocholic acid.

In animals that were administered 5×10^{10} of viable *L. plantarum* WCFS1 rif^R, this bacterium could be recovered from fresh faeces at an average of 1×10^{10} cfu/g faeces. These findings illustrate the relatively high level of persistence and survival of *L. plantarum* WCFS1 in the intestinal tract, which has also been reported earlier (22, 32). Furthermore, *L. plantarum* WCFS1 is able to deconjugate bile salts *in vitro* even when the cells are in an energy-depleted state (Figure 3A), which might be a prerequisite for delivery of Bsh under the potentially nutrient-competitive environment in the intestinal tract. Although *L. plantarum* WCFS1 is capable of *ex vivo* deconjugation of bile salts present in rat bile and in rat intestinal contents (Figure 3B), the delivery of bile salt hydrolase to the small intestine using viable *L. plantarum* had only a minor effect on bile salt composition *in situ*, as exemplified by the amount of deconjugated cholic acid present in the total cholic acid pool (i.e., 3, 6, 1, and 16 % difference in the amount of deconjugated cholic acid present in the total cholic acid pool for SI1,

SI2, SI3, and faeces, respectively, with Student's t-test p-values of 0.27, 0.16, 0.78, and 0.11, respectively; Figure 2A and Figure 1A, chromatogram A2 and A3). Consequently, no effect on mucin excretion in the distal part of the small intestine by the delivery of Bsh by viable *L. plantarum* was found (Figure 4A).

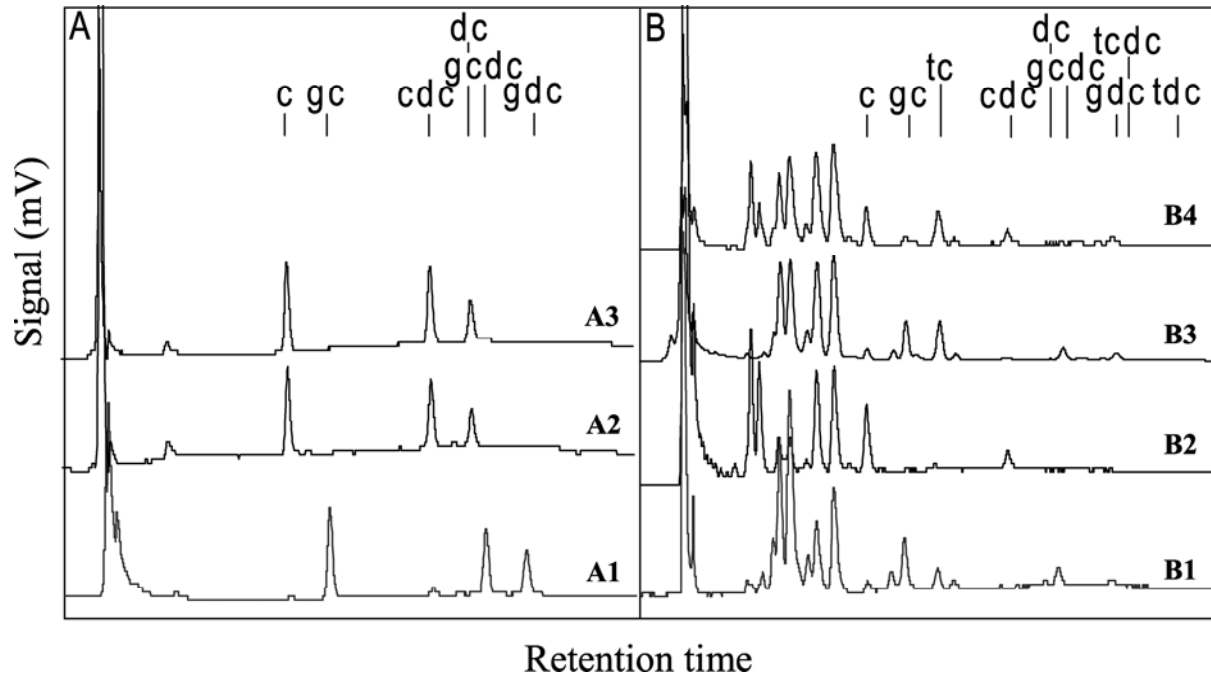


Figure 3. Schematic representation of exemplary HPLC chromatograms of a mixture of synthetic bile salts, rat bile, and intestinal contents. In panel A, a mixture of glycocholic acid (GC), glycochenodeoxycholic acid (GCDC), and glycodeoxycholic acid (GDC) before (A1) and after incubation with energized (A2) and de-energized (A3) *L. plantarum* WCFS1, respectively, is shown. Deconjugation of GC, GCDC, and GDC by these incubations can be readily detected by the appearance of cholic acid (C), chenodeoxycholic acid (CDC) and deoxycholic acid (DC) peaks, respectively. Panel B shows rat bile as it was isolated directly from the bile duct before (B1) and after (B2) *ex vivo* incubation with *L. plantarum* WCFS, and extracts from luminal contents of the distal small intestine before (B3) and after (B4) *ex vivo* incubation with *L. plantarum* WCFS1. Peak positions of bile salts used as a standard are shown. C, cholic acid; TC, taurocholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; TCDC, taurodeoxycholic acid; TDC, taurodeoxycholic acid. Total HPLC retention time was 40 minutes.

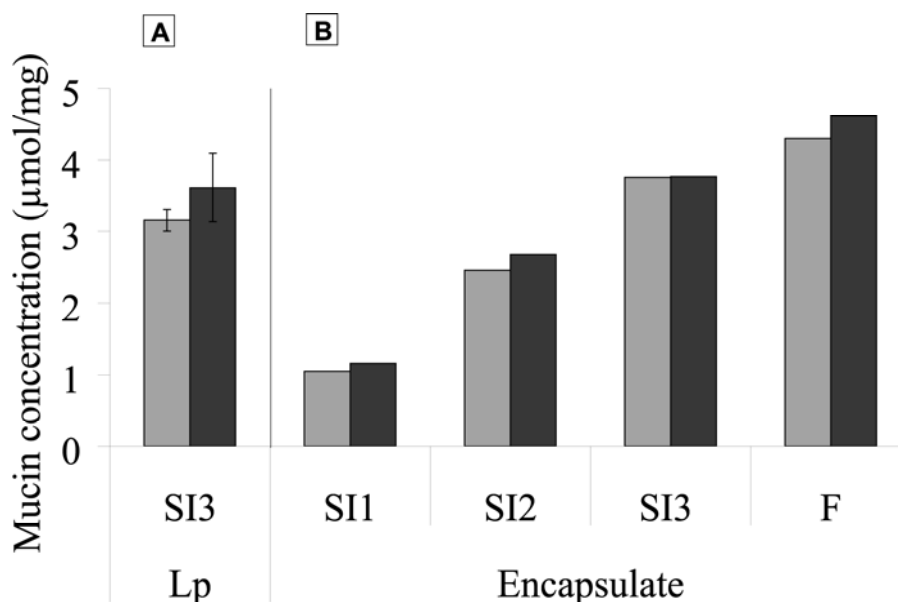


Figure 4. Mucin concentration in the contents of the proximal, middle and anterior part of the small intestine (SI1, SI2, and SI3, respectively) and in faeces (F), expressed in oligosaccharide equivalents released from the mucins during assaying. Panel A: Lp; mucin content in animals fed living (light-grey bars) or heat-killed (dark-grey bars) *L. plantarum* WCFS1. Panel B: Mucin content in pools of samples of animals fed Bsh (light-grey bars) or placebo (dark-grey bars) encapsulate. Error bars represent the SEM of n=9 or n=4 for viable and heat-killed *L. plantarum*, respectively, and n=11 for Bsh or placebo encapsulate.

However, enzyme delivery using viable, wild type bacteria is limited due to constraints in the enzyme expression level that can be reached. Recent studies predicted the activity of *L. plantarum* WCFS1 to be $0.5 \text{ nmol ml}^{-1} \text{ min}^{-1}$ in an *in vitro* assay simulating gastro-intestinal conditions with glycocholic acid as a substrate under small intestinal conditions at an OD_{600} of 3 (Chapter 5). In the present study, 1 ml of a *L. plantarum* culture at an OD_{600} of 30 was fed to the rats, giving rise to a predicted activity of $5 \text{ nmol ml}^{-1} \text{ min}^{-1}$ in the small intestine. Following adherence of *L. plantarum* to a particular area of the small intestine, the bacterial cells would be in contact with intestinal contents for a limited time. In a crude estimation, 30 minutes of contact between the *L. plantarum* cells and intestinal contents passing through is assumed. Thus, in 30 minutes, 150 nmol of bile salts could be deconjugated by *L. plantarum*. When total bile salt production is estimated to be 4 μmol in 30 minutes (16), which is excreted in a constant flow in a rat model, the dose of Bsh activity delivered by *L. plantarum* wild type cells is predicted to be 30 times too low for total deconjugation of the bile salts present in intestinal contents, based on the amount of Bsh activity delivered by *L. plantarum* in our recent *in vitro* intestinal delivery model (Chapter 5). Since the amount of bacteria that can be handled and fed to rats is limited, the level of enzyme delivered by viable bacteria into the intestine can not easily be

increased. When focussed specifically on the delivery of enzymes into the small intestine, genetically modified organisms (GMOs) may yield much higher production levels of enzyme than wild type organisms. However, the use of GMO-derived products is still controversial.

In contrast, the amount of enzyme that can be delivered using microencapsulates is much higher as compared to viable wild type bacteria. However, the delivery of Bsh from microencapsulates is significantly more sensitive to the proteolytic action of digestive enzymes encountered in the small intestine than viable *L. plantarum*, as shown earlier (Chapter 5). Previously, the Bsh activity of the 2.5 g of whey protein-gum arabic microencapsulates, as used in the present study, was shown to be 2 $\mu\text{mol h}^{-1}$ in an *in vitro* model simulating gastro-intestinal conditions, with activity diminishing strongly after an hour due to the activity of pancreatin. Again, in a crude estimation, assuming a passage time of the encapsulates past the bile duct of 30 minutes, 4 μmol of bile salts would be dispersed into the encapsulate bolus. Assuming a total bile salt deconjugation of 2 μmol by the Bsh encapsulates, the activity delivered should have a significant impact on the bile salt composition in the small intestine (~ 50 % deconjugation at the end of the small intestine). However, the protective properties of microencapsulates can be easily destroyed by mastication. This process possibly caused the effect of the delivery of Bsh using microencapsulates on bile salt composition to be minor (i.e., for SI1, SI2, and SI3, 0.5, 0.4, and 0.5 % difference in the amount of cholic acid present in the total cholic acid pool was found, respectively, with Student's t-test p-values of 0.23, 0.14, and 0.22, respectively; Figure 2B and Figure 1B, chromatogram B2 and B3). No effect of the delivery of Bsh by microencapsulates on mucin excretion in faeces or the proximal, middle, or distal part of the small intestine was found (Figure 4B). Furthermore, increasing the incubation time of intestinal contents of rats fed Bsh encapsulates *ex vivo* following dissection of the animals did not increase the bile salt deconjugation level (Figure 5), indicating that the Bsh enzyme likely had been inactivated by digestive enzymes during passage of the intestinal tract and that the limited deconjugation effectuated by the Bsh encapsulates was not due to an insufficient incubation time *in vivo*.

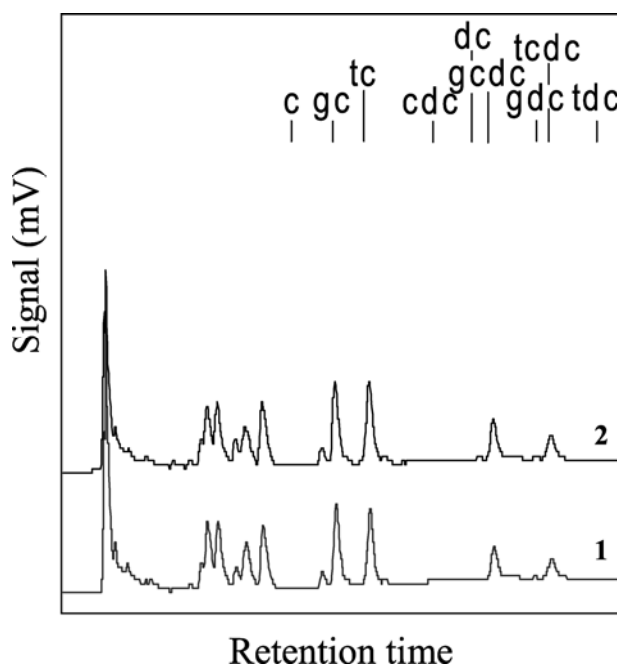


Figure 5. Schematic representation of exemplary HPLC chromatograms of the contents of the proximal small intestine of a rat that was administered Bsh encapsulate before (1) and after (2) additional *ex vivo* incubation, showing that the limited *in vivo* deconjugation conveyed by the Bsh encapsulates was not due to insufficient *in vivo* incubation time. Peak positions of bile salts used as a standard are shown C, cholic acid; GC, glycocholic acid; TC, taurocholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; GCDc, glycochenodeoxycholic acid; GDC, glycodeoxycholic acid; TCDC, taurodeoxycholic acid; TDC, taurodeoxycholic acid. Total HPLC retention time was 40 minutes.

Deconjugated bile salts can be transformed into secondary bile salts such as deoxycholic acid by the natural microbiota present in the intestinal tract, possibly leading to misinterpretation of the bile salt composition, which was based on the pool of the primary bile salt cholic acid present in the *in vivo* samples. Importantly, especially secondary bile salts have been reported to have adverse effects on the host in for example the formation of gallstones and colon cancer (19, 25). To monitor the formation of secondary bile salts following delivery of bile salt hydrolase by *L. plantarum* or microencapsulates, the level of deoxycholic acid and its glycine- and taurine-conjugated counterparts (synthesized in the liver following enterohepatic circulation) in small intestinal contents was determined (Figure 6). However, no significant effect of the delivery of Bsh on the formation of secondary bile salts was found (i.e., for SI1, SI2, and SI3 of rats fed *L. plantarum*, the difference in the ratio of the total deoxycholic acid pool relative to the total cholic acid pool was 0.019, 0.033, and 0.025, respectively, with Student's t-test p-values of 0.81, 0.66, and 0.70, respectively).

Intestinal delivery of bile salt hydrolase activity

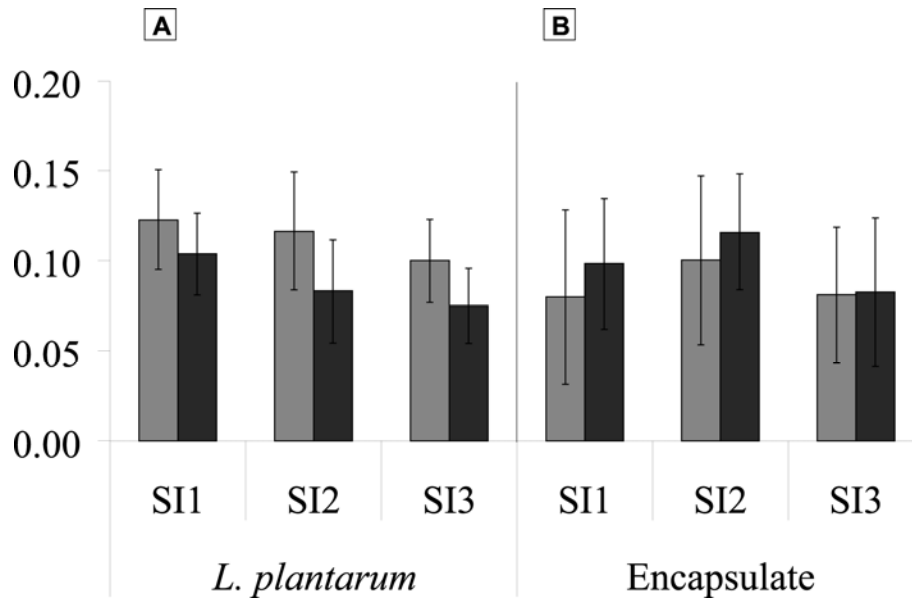


Figure 6. Total deoxycholic acid pool (consisting of deoxycholic acid and its conjugates to glycine and taurine) relative to the total cholic acid pool in the contents of the proximal, middle and anterior small intestine (SI1, SI2, and SI3, respectively) in animals fed *L. plantarum* WCFS1 (panel A; living *L. plantarum*: light-grey bars; heat-killed *L. plantarum*: dark-grey bars) or encapsulate (panel B; Bsh-encapsulate: light-grey bars; placebo encapsulate: dark-grey bars). Error bars represent the SEM of n=9 or n=4 for viable and heat-killed *L. plantarum*, respectively, and n=11 for Bsh or placebo encapsulate.

CONCLUSION

Here we describe the influence of delivery of Bsh using viable *L. plantarum* WCFS1 or whey protein/gum arabic microencapsulates into the small intestine on bile salt composition and mucin concentration in intestinal contents and faeces. Although an appropriate rat model for determination of small intestinal bile salt transformations was implemented, no effect of delivery of Bsh on the intestinal bile salt composition or luminal mucin concentration was detected. These results may indicate that the physiological relevance and magnitude of bile salt hydrolase activity of probiotics introduced in the small intestine is limited, alleviating concerns on the bile salt hydrolase activity produced by probiotic strains, especially lactobacilli. However, other strains commonly used as probiotics include bifidobacterial strains, which have previously been shown to generally express Bsh activity at a higher level as compared to lactobacilli (28), and therefore might have a more pronounced effect on bile metabolism *in vivo*. The rat model and methodology described here could be used to effectively investigate the possible effects of the administration of bifidobacteria on the host.

ACKNOWLEDGEMENTS

The authors of this manuscript would like to thank Roger Bongers for assistance during the dissection of animals in the encapsulate experiment and Denise Jonker for her valuable technical contribution to the mucin assays.

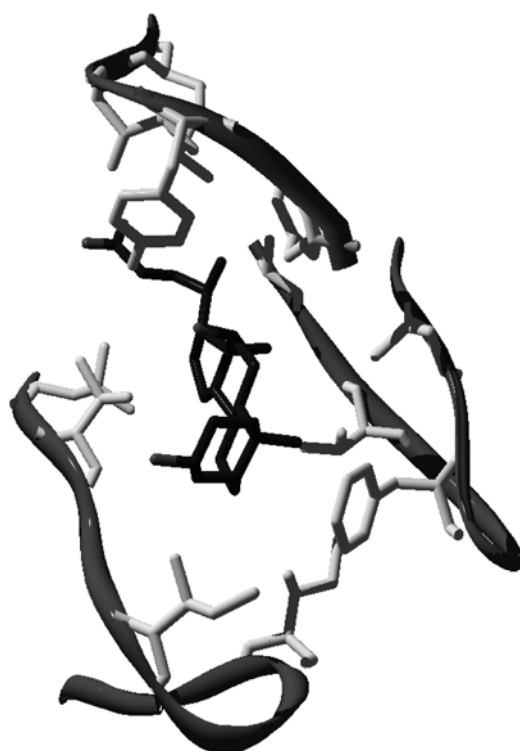
REFERENCES

1. **Agellon, L. B.** 2002. p. 433-448. *In* D. E. a. V. Vance, J.E. (ed.), Biochemistry of Lipids, Lipoproteins and Membranes, 4th ed. Elsevier Science B.V.
2. **Batta, A. K., G. Salen, and S. Shefer.** 1984. Substrate specificity of cholyglycine hydrolase for the hydrolysis of bile acid conjugates. *J Biol Chem* **259**:15035-9.
3. **Begley, M., C. Hill, and C. G. Gahan.** 2006. Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol.* **72**:1729-38.
4. **Begley, M., R. D. Sleator, C. G. Gahan, and C. Hill.** 2005. Contribution of three bile-associated loci, *bsh*, *pva*, and *btlB*, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. *Infect. Immun.* **73**:894-904.
5. **Bhopatkar, D., A. K. Anal, and W. F. Stevens.** 2005. Ionotropic alginate beads for controlled intestinal protein delivery: effect of chitosan and barium counter-ions on entrapment and release. *J. Microencapsul.* **22**:91-100.
6. **Bovee-Oudenhoven, I. M., D. S. Termont, P. J. Heidt, and R. Van der Meer.** 1997. Increasing the intestinal resistance of rats to the invasive pathogen *Salmonella enteritidis*: additive effects of dietary lactulose and calcium. *Gut* **40**:497-504.
7. **Crowther, R. S., and R. F. Wetmore.** 1987. Fluorometric assay of O-linked glycoproteins by reaction with 2-cyanoacetamide. *Anal. Biochem.* **163**:170-4.
8. **De Smet, I., L. Van Hoorde, M. Vande Woestyne, H. Christiaens, and W. Verstraete.** 1995. Significance of bile salt hydrolytic activities of lactobacilli. *J. Appl. Bacteriol.* **79**:292-301.
9. **Dekker, R., Meer, R. van der, Olieman, C.** 1991. Sensitive pulsed amperometric detection of free and conjugated bile acids in combination with gradient reversed-phase HPLC. *Chromatographia* **31**:549-553.
10. **Floris, R., Bodnár, I, Weinbreck, F, Altin A. C.** 2007. Dynamic rearrangement of disulfide bridges influences solubility of whey protein coatings. Accepted for publication in *Int. Dairy J.*
11. **Hardison, W. G., and J. H. Proffitt.** 1977. Influence of hepatic taurine concentration on bile acid conjugation with taurine. *Am. J. Physiol.* **232**:E75-9.
12. **Huyghebaert, N., A. Vermeire, P. Rottiers, E. Remaut, and J. P. Remon.** 2005. Development of an enteric-coated, layered multi-particulate formulation for ileal delivery of viable recombinant *Lactococcus lactis*. *Eur. J. Pharm. Biopharm.* **61**:134-41.
13. **Ide, T., and M. Horii.** 1989. Predominant conjugation with glycine of biliary and lumen bile acids in rats fed on pectin. *Br. J. Nutr.* **61**:545-57.
14. **Ide, T., M. Horii, K. Kawashima, and T. Yamamoto.** 1989. Bile acid conjugation and hepatic taurine concentration in rats fed on pectin. *Br. J. Nutr.* **62**:539-50.
15. **Ide, T., M. Horii, T. Yamamoto, and K. Kawashima.** 1990. Contrasting effects of water-soluble and water-insoluble dietary fibers on bile acid conjugation and taurine metabolism in the rat. *Lipids* **25**:335-40.
16. **Kararli, T. T.** 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm. Drug Dispos.* **16**:351-80.

17. Kirby, L. C., Klein, R. A., Coleman, J. P. 1995. Continuous spectrophotometric assay of conjugated bile acid hydrolase. *Lipids* **30**:863-867.
18. Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen. 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. USA* **100**:1990-5.
19. Klinkspoor, J. H., R. Kuver, C. E. Savard, D. Oda, H. Azzouz, G. N. Tytgat, A. K. Groen, and S. P. Lee. 1995. Model bile and bile salts accelerate mucin secretion by cultured dog gallbladder epithelial cells. *Gastroenterology* **109**:264-74.
20. Klinkspoor, J. H., K. S. Mok, B. J. Van Klinken, G. N. Tytgat, S. P. Lee, and A. K. Groen. 1999. Mucin secretion by the human colon cell line LS174T is regulated by bile salts. *Glycobiology* **9**:13-9.
21. Lambert, J. M., R. S. Bongers, and M. Kleerebezem. 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **73**:1126-35.
22. Pavan, S., P. Desreumaux, and A. Mercenier. 2003. Use of mouse models to evaluate the persistence, safety, and immune modulation capacities of lactic acid bacteria. *Clin. Diagn. Lab. Immunol.* **10**:696-701.
23. Ramadas, M., W. Paul, K. J. Dileep, Y. Anitha, and C. P. Sharma. 2000. Lipoinsulin encapsulated alginate-chitosan capsules: intestinal delivery in diabetic rats. *J. Microencapsul.* **17**:405-11.
24. Reeves, P. G., F. H. Nielsen, and G. C. Fahey, Jr. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition *ad hoc* writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **123**:1939-51.
25. Ridlon, J. M., D. J. Kang, and P. B. Hylemon. 2006. Bile salt biotransformations by human intestinal bacteria. *J. Lipid Res.* **47**:241-59.
26. Shimotoyodome, A., S. Meguro, T. Hase, I. Tokimitsu, and T. Sakata. 2000. Decreased colonic mucus in rats with loperamide-induced constipation. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **126**:203-12.
27. Sjovall, J. 1959. Dietary glycine and taurine on bile acid conjugation in man; bile acids and steroids 75. *Proc. Soc. Exp. Biol. Med.* **100**:676-8.
28. Tanaka, H., Doesburg, K., Iwasaki, T., Mierau, I. 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. *J. Dairy Sci.* **82**:2530-2535.
29. Tannock, G. W., M. P. Dashkevich, and S. D. Feighner. 1989. Lactobacilli and bile salt hydrolase in the murine intestinal tract. *Appl. Environ. Microbiol.* **55**:1848-51.
30. Tannock, G. W., A. Tangerman, A. Van Schaik, and M. A. McConnell. 1994. Deconjugation of bile acids by lactobacilli in the mouse small bowel. *Appl. Environ. Microbiol.* **60**:3419-20.
31. Van der Meer, R., J. W. Welberg, F. Kuipers, J. H. Kleibeuker, N. H. Mulder, D. S. Termont, R. J. Vonk, H. T. De Vries, and E. G. De Vries. 1990. Effects of supplemental dietary calcium on the intestinal association of calcium, phosphate, and bile acids. *Gastroenterology* **99**:1653-9.
32. Vesa, T., P. Pochart, and P. Marteau. 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. *Aliment. Pharmacol. Ther.* **14**:823-8.
33. Weinbreck, F., M. Minor, and C. G. de Kruif. 2004. Microencapsulation of oils using whey protein/gum Arabic coacervates. *J. Microencapsul.* **21**:667-79.

Chapter 7.

General discussion and concluding remarks.



7. General discussion and concluding remarks

Bacterial bile salt hydrolase activity appears to play an important role in both bacterial survival and persistence in the intestinal tract and in host intestinal physiology. In this thesis, various aspects of bile salt hydrolase activity were investigated in both bacteria and host model systems. Focussing on bacteria, the functionality of conjugated bile acid hydrolase (CBAH) superfamily members in Gram-positive bacteria was investigated, with an emphasis on the organism *Lactobacillus plantarum* WCFS1 used as a model for lactobacilli. Focussing on the host, the delivery of bile salt hydrolase into the small intestine was investigated both *in vitro* and *in vivo*. Below, the tools and most important results obtained during this PhD project are placed in a general context, with specific attention for their impact and future research directions.

Improved annotation of CBAH superfamily members. An advanced bioinformatics approach was developed and applied for improved annotation of CBAH superfamily members in Gram-positive bacteria (**Chapter 2**). For this purpose, various *in silico* analysis methods were combined, including both phylogenetic clustering and 3D-modeling techniques, in addition to the more conventional method of sequence alignment. These resulted in the development of novel and discriminative hidden Markov models (HMMs) that can be used to distinguish between the homologous Bsh and Pva-family members, which appeared not feasible using the available and commonly used conserved motif databases. Following this advanced analysis, we propose re-annotation of eight *Lactobacillus* genes that belong to the CBAH superfamily from *bsh* into *pva*, including three of the four genes annotated as redundant *bsh* genes in *L. plantarum* (i.e., *bsh2*, *bsh3*, and *bsh4*).

The hypothesis that Bsh activity is important for survival or persistence of bacteria in the host was reinforced here by the fact that all strains that were represented only in the Bsh-cluster are typical gut-related micro-organisms (i.e. Bifidobacteria, *Enterococcus faecalis* and *faecium*, and *Lactobacillus acidophilus* species), while strains represented only in the Pva-cluster were not. This is in agreement with earlier findings, where a strong correlation was found between the habitat of a specific bacterial species or strain and Bsh activity (31). Furthermore, all experimentally verified Bsh and Pva enzymes were clustered correctly in their corresponding gene family (i.e., the Bsh- or Pva-family), supporting the phylogenetic clustering method used here.

Another important finding was the observation that the members of the Pva- and Bsh-clusters were not only found to be phylogenetically, but also structurally different (**Chapter 2**). Homology modelling and sequence alignment showed clear differences in the 3D-structure of the substrate binding pockets of Bsh- and Pva-enzymes. The benefits of the determination and

implementation of 3D protein structures – both those obtained experimentally and via homology modelling – in elucidating the biochemical function of proteins is clearly shown in this thesis. Recognition of the value of protein structure determination has led to the initiation of large scale activities aiming at the complete structural characterization of conserved biochemical pathways and processes (6), involving high-throughput protein production, crystallisation, homology modelling and functional annotation.

Importantly, the methodology used here does not need to be limited to the CBAH superfamily. The rapid expansion of genome sequence data calls for improved annotation methods, since many biologically important gene families show functional or genetic redundancy or are simply notoriously difficult to annotate. Annotation based only on sequence similarity usually does not lead to a reliable prediction of functionality, but to the classification of the putative protein into a specific protein superfamily with common activities. For example, the annotation of transporters is often difficult because of the existence of large and complex transporter gene superfamilies, such as the ATP-binding cassette (ABC) (8), ATPase (20) and major facilitator (MFS) (26) superfamilies, and the presence of multiple transporter gene paralogs in many organisms. Phylogenetic profiling provided an insight into the differences in membrane transport in prokaryotes and eukaryotes (18). However, the incorporation of data on experimentally verified enzymes could provide additional and highly valuable information by enabling the annotation of an experimentally verified functionality to the clusters found. Indeed, the use of experimental data in phylogenetic profiling is crucial for interpretation and validation of the phylogenetic clusters. Furthermore, for some transporters, the crystal structure is known, providing opportunities for gathering additional information on their functionality. For example, several putative proteins in *L. plantarum* that have been annotated as manganese transporters are not highly homologous in sequence, but are predicted to be functionally redundant. Phylogenetic profiling was used to refine the annotation of these sequences (C. Francke, unpublished results). Other examples of protein superfamilies where the methodology described here could be implemented include the NADH oxidase family (PeroxiBase; (21)), and the thioredoxin superfamily (2). More specific examples include the four genes annotated as pyruvate oxidase (*pox*) in *L. plantarum*, where only two genes were found to encode true pyruvate oxidases (11, 12), and the four genes annotated as lactate dehydrogenase (*ldh*) in *Lactococcus lactis*; for these proteins, the crystal structure is also known (19, 24, 36).

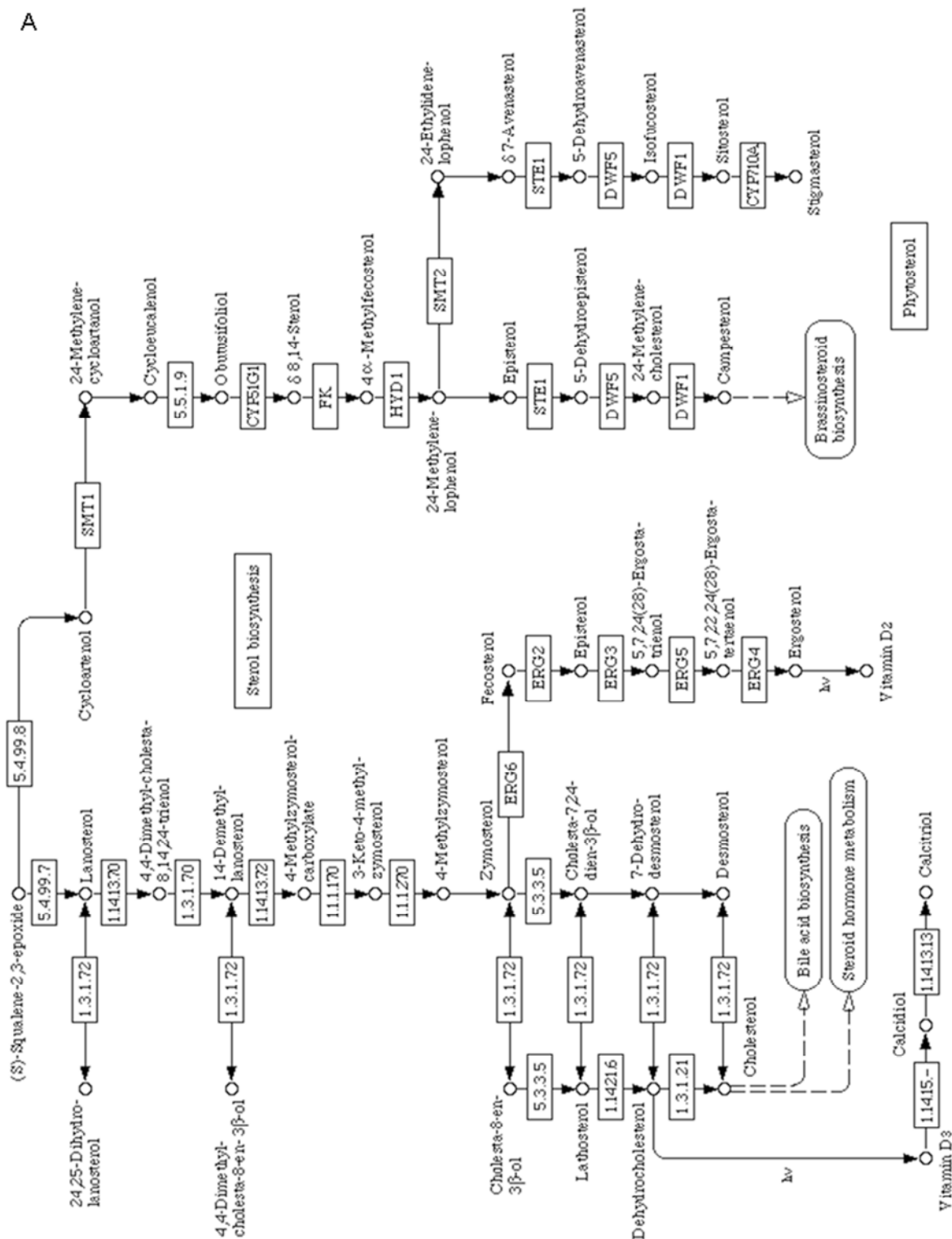
Exploring the functionality of redundant genes. Clearly, even when using improved *in silico* annotation methods as described in this thesis, there appear to be many functionally redundant proteins. However, from an evolutionary perspective, conserved genes should have a certain functionality. Thus, many putative proteins predicted to be redundant may merely have a function unknown to us. Furthermore, *in silico* predictions will always need to

be validated by experimental evidence. The unravelling of the functionality of each of the separate redundant genes in a single organism requires the generation of multiple deletion strains. For this purpose, a Cre-*lox*-based toolbox for the construction of multiple deletions and selectable-marker removal in Gram-positive organisms was designed and implemented in *L. plantarum* WCFS1 (**Chapter 3**). The generated Cre-*lox*-based mutagenesis system appeared to be highly effective in multiple gene replacements and deletions in a single genetic background, as exemplified by successive mutagenesis of the genetically closely linked loci *melA* and *lacS2* in *L. plantarum* WCFS1, where essentially all of the clones that were analysed had undergone correct Cre-*lox* resolution.

Previously, the four CBAH family members *bsh1*, *bsh2*, *bsh3*, and *bsh4* of *L. plantarum* WCFS1 were predicted to be redundant genes. However, *bsh2*, *bsh3*, and *bsh4* were proposed to be re-annotated to penicillin acylase-related genes according to our *in silico* analyses. Therefore, it was interesting to investigate their true functionality. For this purpose, heterologous overexpression strains and multiple *bsh*-deletion strains were constructed, the latter using the Cre-*lox* toolbox (**Chapter 4**). In agreement with our *in silico* prediction, *bsh1* was shown to be the major bile salt hydrolase in *L. plantarum*, and was involved in glycodeoxycholic acid tolerance.

In addition, *bsh2*, *bsh3*, and *bsh4* appeared to be conserved among *L. plantarum* strains, suggesting a biologically important function that, however, remains unclear. It is tempting to speculate that their function is related to acylase activity with penicillin-like chemicals as substrate. Since the *in vivo* role of penicillin acylase is unknown, Bsh2, Bsh3 and Bsh4 may play a role in acylation of compounds other than the substrates tested in this work, such as additional phenylacetic acid amide derivatives, as suggested by the capability of penicillin acylases to cleave these substrates in addition to penicillins (37). Phenylacetic acid derivatives would be available to *L. plantarum in vivo*, since they are formed on fermenting plants. Lactic acid bacteria are commonly found on a wide range of fermenting plant material, such as silage, cabbage (sauerkraut and Korean kirachi), and various pickles (10, 29). Plants synthesise a large amount of phenols and phenolic acids, known for their antimicrobial activity, such as cinnamic and caffeic acids (for review, see (7)). Thus, acylation activity on plant phenylacetic acid derivatives by bacterial CBAH superfamily proteins could well serve as a detoxification mechanism. In addition, many types of plants synthesise a large range of isoprenoids via the mevalonate pathway (for example, see Figure 1A; (30)), which in their backbone show a significant structural resemblance with bile salts (Figure 1B – D). Isoprenoids are synthesised from oxosqualene ((S)-squalene-2,3-epoxide; Figure 1A), which is converted into lanosterol, leading to the pathway involved in synthesis of cholesterol, and cycloartenol, which leads to the synthesis of phytosterols. Interestingly, some oxosqualene, lanosterol, and cycloartenol derivatives called

saponins are bactericidal (7, 39, 40). In plants, a wide array of saponins has been found that is still expanding (39). Thus, putative amide-derivatives of plant isoprenoids may serve as an alternative substrate for Bsh-family members, possibly in the detoxification of saponins.



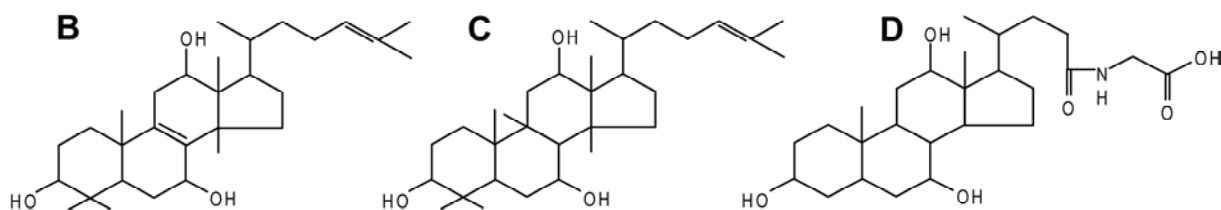


Figure 1. Panel A. Part of the isoprenoid synthesis pathway (derived from the KEGG database (15)), showing the vast amount of sterols synthesised in this pathway alone. Panel B, C, and D show the molecular structure of lanosterol, cycloartenol, and the bile salt glycocholic acid, respectively.

The putative activity of CBAH superfamily members on secondary plant metabolites cannot easily be assessed due to limited commercial availability of the substrates and to the vast amount of different plant metabolites that exists. However, the availability of the defined set of Bsh mutants in *L. plantarum* will allow for further studies on their functionality even in the absence of purified substrates.

Delivery of Bsh to the host intestine. It has been reported that intestinal lactobacilli are of importance for *in vivo* bile salt deconjugation in the small intestine (4, 32, 33). Currently, there is an increasing interest in the use of lactobacilli as a probiotic to positively influence health status. Because of the alleged role of Bsh in survival and persistence of bacterial strains in the intestinal tract, a commonly used criterion for the selection of candidate probiotic strains is their capability to hydrolyse bile salts. However, the administration of large amounts of bile salt-hydrolysing probiotic bacteria could potentially result in a shift in intestinal bile salt composition that might be associated with an undesirable impact on the host, such as the formation of gallstones or promotion of colon cancer. Therefore, the impact on host physiology of Bsh delivery into the small intestine deserves attention.

To investigate the influence of small intestinal delivery of bile salt hydrolase on the host, the lactic acid bacterium *L. plantarum* WCFS1 was used as a model for the administration of bile salt-hydrolysing activity. *In vitro* experiments showed that *L. plantarum* WCFS1 was capable to withstand gastric conditions as well as pancreatin challenge (**Chapter 5**), making it an ideal vehicle for *in vivo* delivery of enzymes. In a placebo-controlled human intervention study, *L. plantarum* WCFS1 was capable of deconjugating bile salts in healthy volunteers. In this study, *L. plantarum* or placebo was perfused into the small intestine for one hour using a catheter that enabled injection of a fluid 5 cm distal to the pylorus, and aspiration of luminal contents at 40 cm distal to the injection port. This catheter was inserted during a gastroduodenoscopy procedure, as described previously (35). Following placebo perfusion, the bile salts in the aspirated luminal contents consisted of the normal human conjugated bile salts glyco- and tauro-cholic acid, -chenodeoxycholic acid and -deoxycholic acid, as determined by HPLC analysis (Figure 2, chromatogram 1). In the same

volunteer, when perfused on a separate occasion with *L. plantarum*, small but significant HPLC peaks of the deconjugated forms of these bile acids appeared (i.e., free cholic, chenodeoxycholic, and deoxycholic acid), clearly showing the capability of *L. plantarum* WCFS1 to deconjugate bile salts *in vivo* (Figure 2). Comparative transcriptome analysis of mucosal biopsies taken after placebo or *L. plantarum* perfusion did not show a significant differential response related to Bsh activity as a consequence of the *L. plantarum* perfusion (Fred Troost, unpublished data). This could be due to the fact that these biopsies were obtained from the horizontal part of the duodenum, which is a position more proximal to the stomach than the locus where the luminal contents used for determination of the intestinal bile salt composition were aspirated. Consequently, the bile salt deconjugation level at the site where the biopsies were taken is likely to be much less exposed to deconjugated bile salts as compared to regions further down the small intestine, based on the shorter exposure of the native bile salts to the *L. plantarum* Bsh activity.

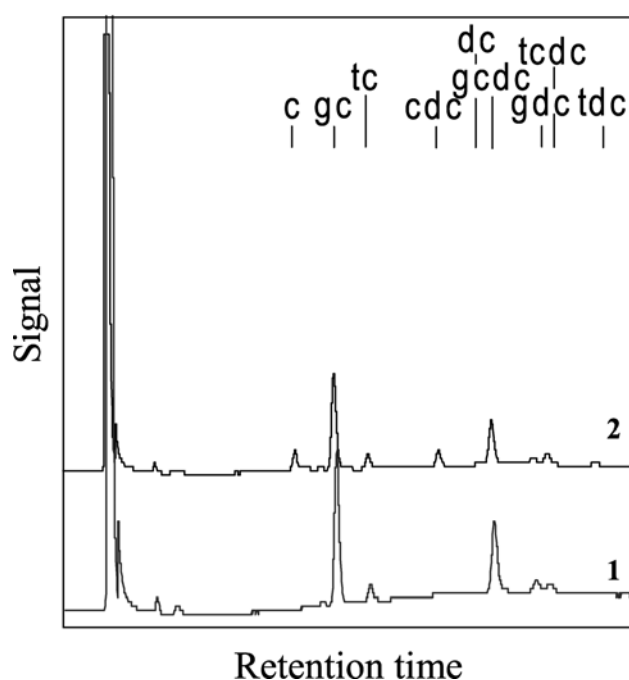


Figure 2. Schematic representation of exemplary HPLC chromatograms of human small intestinal samples after perfusion with placebo (1) or with *L. plantarum* (2). Peak positions of bile salts used as a standard are shown C, cholic acid; GC, glycocholic acid; TC, taurocholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; GCDc, glycochenodeoxycholic acid; GDC, glycodeoxycholic acid; TCDC, taurodeoxycholic acid; TDC, taurodeoxycholic acid.

To establish the impact of Bsh on the host, several studies were performed using animal models. In the rat model we used to determine the impact of

delivery of Bsh on host physiology (**Chapter 6**), the human situation was approximated as much as possible by a low calcium and high fat diet, simulating the composition of a Western human diet (38). Rats naturally produce bile salts mostly conjugated to taurine, whereas in humans, bile salts are mostly conjugated to glycine (see above, and Figure 2). In addition, most Bsh enzymes prefer glycine-conjugated over taurine-conjugated bile salts (3). Therefore, in the rat model implemented here, the conjugation of bile salts was shifted from predominantly taurine to glycine by inclusion of pectin and cholesterol in the diet. The developed animal model appeared to be an excellent tool to measure the impact of delivery of bile salt hydrolase in the small intestine in a conventional animal, since background bile salt deconjugation levels were very low. This circumvented the need to use germ-free animals, which is a system that is far from the normal situation. In the developed animal model, bile salt deconjugation appeared to occur mostly in the colon. Although *L. plantarum* WCFS1 is capable of *ex vivo* deconjugation of bile salts present in rat bile and in rat intestinal contents, the delivery of bile salt hydrolase to the small intestine using viable *L. plantarum* merely induced a trend effect on bile salt composition *in vivo* (**Chapter 6**). As described earlier, one of the possible mechanisms implemented by the host in response to generation of the more toxic deconjugated bile salts is upregulating the production of mucin. (16). However, the delivery of Bsh by *L. plantarum* had no effect on host mucin excretion.

In an additional study, the effect of Bsh delivery by *L. plantarum* on the transcriptome profile of the distal small intestine was determined by co-colonising germ-free mice fed a Western-style diet with the Bsh-negative *Bacteroides thetaiotamicron* and wild type *L. plantarum* or its four-fold *bsh*-mutant derivative, that was constructed earlier (**Chapter 4**). Affymetrix genechip analysis of the distal SI of these mice revealed that the absence of Bsh production in the *L. plantarum* *bsh*-mutant strain changed the expression of 298 genes (i.e., 172 genes downregulated, and 126 genes upregulated). Among those genes, a group of 22 genes related to host lipid metabolism was regulated moderately but significantly, of which 18 genes were downregulated, suggesting an effect of delivery of Bsh on host lipid metabolism. However, we found no effect of colonisation of wild-type *L. plantarum* on bile salt composition as compared to its *bsh*-mutant derivative (Figure 3). However, we can not rule out the possibility that a small amount of deconjugated bile salts formed by *L. plantarum* WCFS1 in the intestinal lumen escaped detection due to fast uptake by the intestinal epithelium into the enterohepatic cycle. Since the major bile salt hydrolase of *L. plantarum* WCFS1, Bsh1, showed a preference towards glycine- over taurine-conjugated substrates, the bile salt hydrolase activity *in vivo* on the mainly taurine-conjugated murine bile salts (Figure 3) may have been limited. Possibly, the effect of Bsh on host physiology with a Bsh that displays higher activity levels towards taurine-conjugated bile salts could have a larger impact on the bile salt composition in a conventional rat or mouse model.

However, most Bshs that have been described show a preference for glycine- over taurine-conjugated bile salts (3).

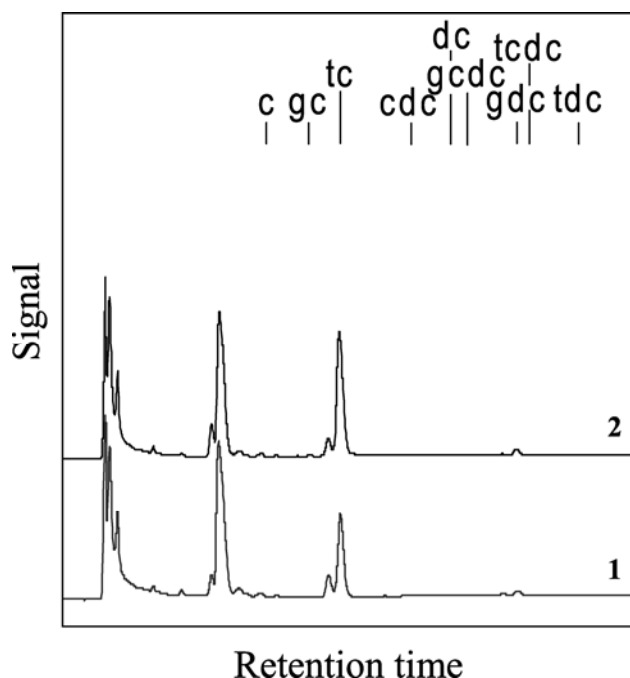


Figure 3. Schematic representation of exemplary HPLC chromatograms of the contents of the proximal small intestine of a germ-free mouse that was administered placebo (1) or *L. plantarum* (2). Peak positions of bile salts used as a standard are shown C, cholic acid; GC, glycocholic acid; TC, taurocholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; GCDC, glycochenodeoxycholic acid; GDC, glycodeoxycholic acid; TCDC, taurodeoxycholic acid; TDC, taurodeoxycholic acid.

Clearly (see above), the rat model we used in this thesis (**Chapter 6**) that produced mainly glycine-conjugated bile salts may be preferable for determination of the impact of bile salt hydrolase on host physiology. Although some animal models produce mainly glycine-conjugated bile salts (e.g., guinea pig), their applicability is restricted due to limited availability of suitable transcriptome analysis platforms. In addition, it would be interesting to use *Lactobacillus*-free mice that have been described earlier (32, 33), to determine the impact of *L. plantarum* WCFS1 on bile salt deconjugation *in vivo*. In this animal model, lactobacilli were previously shown to be responsible for most (i.e., 90 %) of the Bsh activity in the small intestine (32).

Since enzyme delivery using viable, wild type bacteria is limited due to constraints in the enzyme expression level that can be reached, an alternative for bacterial delivery might be provided by use of enteric encapsulates, where the amount of enzyme that can be incorporated is virtually unlimited. Therefore, the properties of delivery using food-grade protein-gum arabic encapsulates were determined, using Bsh as a model enzyme. *In vitro* experiments showed that the microencapsulates gave rise to highly effective protection of the enzyme under

gastric conditions (**Chapter 5** – see above). Moreover, enzyme release under intestinal conditions appeared to be very efficient, although in contrast to delivery by *L. plantarum*, the Bsh activity decreased in time due to proteolytic degradation in presence of pancreatin (**Chapter 5**). However, when used in our rat model, no effect of the delivery of Bsh by microencapsulates on bile salt composition or mucin excretion was detected.

The results obtained for intestinal delivery of Bsh may indicate that the physiological relevance and magnitude of bile salt hydrolase activity of the natural microbiota and probiotics in the small intestine is not very high, alleviating concerns on the bile salt hydrolase activity produced *in vivo* by probiotic strains, especially lactobacilli. However, other strains commonly used as probiotics include bifidobacterial strains, which have previously been shown to generally express Bsh activity at a higher level as compared to lactobacilli (31), and therefore might have a more pronounced effect on bile metabolism *in vivo*. The rat model and methodology described here could be used to effectively investigate the possible effects of the administration of bifidobacteria on the host.

Delivery of enzymes using genetically modified organisms. When comparing intestinal delivery by viable bacteria and microencapsulates, delivery of therapeutics using bacteria in treating or preventing disease may be preferable due to their natural adjuvant activity and low immunogenicity (22, 23) (Peter van Baarlen, unpublished results). In addition, the implementation of viable bacteria as a delivery vehicle is attractive because of their food-grade nature and existing association with health by the general public.

As a solution for the limited expression levels that can be obtained in wild type bacteria, genetically modified organisms (GMOs) can be used. For example, the implementation of genetical engineering allows for the expression of exogenous gene products (for review, see (13)); e.g., several organisms such as *Lactococcus lactis*, *L. plantarum*, and *Streptococcus gordonii* have been used for delivery of therapeutics *in vivo*. In *Lactococcus lactis*, the cytokine IL-10, which is a powerful anti-inflammatory cytokine that plays a central role in downregulation of inflammatory cascades (1), was expressed for use in treatment in inflammatory bowel disease. In mice with dextran sulphate sodium (DSS)-induced enterocolitis, inflammation was reduced by IL-10 delivered by *Lactococcus lactis* (27, 28). Furthermore, the dose of IL-10 required could be decreased a 1000-fold when using localised delivery by viable bacteria when compared to standard non-localised systemic treatment (28). In addition, the intake of trefoil factor (TFF)-secreting *Lactococcus lactis* was shown to convey repair of inflammatory damage to the epithelium of DSS-induced colitis. TFFs are known to be important in the protection and repair of the intestinal epithelium (34). Other examples include the expression of a model antigen, consisting of an epitope derived from the HIV-virus, in *L. plantarum* (14), where high expression levels could be reached. Furthermore, lipid digestion

could be enhanced in pigs by the expression of a lipase in *Lactococcus lactis* (9). Another application of GMO technology was the reduction of caries by the production of anti-*Streptococcus mutans* antibodies by *Lactobacillus zeae* (17) in rats. In the field of vaccination, tetanus toxin fragment C (TTFC) has been expressed in both *Lactococcus lactis* and *L. plantarum* (5, 25), yielding protection against tetanus following mucosal immunization regimes. Cell wall mutants of *Lactococcus lactis* and *L. plantarum* that contained an alanine racemase (*alr*) mutation were shown to be more effective in immunisation, possibly due to improved release of antigen from the bacterial cells. However, since alanine racemase is an essential gene in these organisms (14), the use of *alr* mutants could have a second, important application in containment of the bacterial strains when used outside the laboratory. To date, biological safety of GMOs is still subject to debate. Recently, the first clinical trial using live IL-10 expressing *Lactococcus lactis* that is contained by the deletion of the essential gene *thyA* has been approved and carried out in The Netherlands (28), which is an important step forward in the introduction of GMOs in medical applications.

Concluding remarks. The work presented in this thesis led to the improvement and refinement of the annotation of CBAH superfamily members in Gram-positive bacteria into bile salt hydrolases and penicillin acylase-related enzymes. However, several bacteria appeared to be redundant for bile salt hydrolase or penicillin acylase-related functionality, such as *L. plantarum* WCFS1, which contains four genes (*bsh1* to *bsh4*) originally annotated as bile salt hydrolase. To enable investigation of genes predicted to be redundant, a Cre-*lox* toolbox for the construction of multiple deletions and selectable-marker removal in Gram-positive organism was designed and implemented in *L. plantarum* WCFS1. Analysis of the functionality of Bsh1, Bsh2, Bsh3, and Bsh4 of *L. plantarum* WCSF1 revealed that Bsh1 is the major bile salt hydrolase in this strain, whereas Bsh2, Bsh3, and Bsh4 appear to be penicillin acylase-related. The delivery of bile salt hydrolase activity to the small intestine using viable *L. plantarum* was compared to delivery using Bsh-whey protein/gum arabic microencapsulates *in vitro*. However, no effect of delivery of Bsh on the intestinal bile salt composition or mucin excretion in a rat model was found. In contrast, initial human experiments showed that *L. plantarum* WCFS1 has the capacity to deconjugate human bile salts *in vivo*.

REFERENCES

1. **Asadullah, K., W. Sterry, and H. D. Volk.** 2003. Interleukin-10 therapy--review of a new approach. *Pharmacol. Rev.* **55**:241-69.
2. **Aslund, F., and J. Beckwith.** 1999. The thioredoxin superfamily: redundancy, specificity, and gray-area genomics. *J. Bacteriol.* **181**:1375-9.
3. **Begley, M., C. Hill, and C. G. Gahan.** 2006. Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol.* **72**:1729-38.

4. **Bongaerts, G. P., R. S. Severijnen, A. Tangerman, A. Verrips, and J. J. Tolboom.** 2000. Bile acid deconjugation by *Lactobacilli* and its effects in patients with a short small bowel. *J. Gastroenterol.* **35**:801-4.
5. **Bron, P. A., C. Granette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. *J. Bacteriol.* **186**:5721-9.
6. **Burley, S. K., and J. B. Bonanno.** 2002. Structural genomics of proteins from conserved biochemical pathways and processes. *Curr Opin Struct Biol* **12**:383-91.
7. **Cowan, M. M.** 1999. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **12**:564-82.
8. **Davidson, A. L., and J. Chen.** 2004. ATP-binding cassette transporters in bacteria. *Annu. Rev. Biochem.* **73**:241-68.
9. **Drouault, S., C. Juste, P. Marteau, P. Renault, and G. Corthier.** 2002. Oral treatment with *Lactococcus lactis* expressing *Staphylococcus hyicus* lipase enhances lipid digestion in pigs with induced pancreatic insufficiency. *Appl. Environ. Microbiol.* **68**:3166-8.
10. **Gardner, N. J., T. Savard, P. Obermeier, G. Caldwell, and C. P. Champagne.** 2001. Selection and characterization of mixed starter cultures for lactic acid fermentation of carrot, cabbage, beet and onion vegetable mixtures. *Int. J. Food Microbiol.* **64**:261-75.
11. **Goffin, P., F. Lorquet, M. Kleerebezem, and P. Hols.** 2004. Major role of NAD-dependent lactate dehydrogenases in aerobic lactate utilization in *Lactobacillus plantarum* during early stationary phase. *J. Bacteriol.* **186**:6661-6.
12. **Goffin, P., L. Muscariello, F. Lorquet, A. Stukkens, D. Prozzi, M. Sacco, M. Kleerebezem, and P. Hols.** 2006. Involvement of pyruvate oxidase activity and acetate production in the survival of *Lactobacillus plantarum* during the stationary phase of aerobic growth. *Appl. Environ. Microbiol.* **72**:7933-40.
13. **Hanniffy, S., U. Wiedermann, A. Repa, A. Mercenier, C. Daniel, J. Fioramonti, H. Tlaskolova, H. Kozakova, H. Israelsen, S. Madsen, A. Vrang, P. Hols, J. Delcour, P. Bron, M. Kleerebezem, and J. Wells.** 2004. Potential and opportunities for use of recombinant lactic acid bacteria in human health. *Adv. Appl. Microbiol.* **56**:1-64.
14. **Hols, P., P. Slos, P. Dutot, J. Reymund, P. Chabot, B. Delplace, J. Delcour, and A. Mercenier.** 1997. Efficient secretion of the model antigen M6-gp41E in *Lactobacillus plantarum* NCIMB 8826. *Microbiology* **143 (Pt 8)**:2733-41.
15. **Kanehisa, M., and S. Goto.** 2000. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **28**:27-30.
16. **Klinkspoor, J. H., K. S. Mok, B. J. Van Klinken, G. N. Tytgat, S. P. Lee, and A. K. Groen.** 1999. Mucin secretion by the human colon cell line LS174T is regulated by bile salts. *Glycobiology* **9**:13-9.
17. **Lehner, T.** 1985. Immunization against dental caries. *Vaccine* **3**:65-8.
18. **Molenaar, D., F. Bringel, F. H. Schuren, W. M. de Vos, R. J. Siezen, and M. Kleerebezem.** 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. *J. Bacteriol.* **187**:6119-27.
19. **Muller, Y. A., G. Schumacher, R. Rudolph, and G. E. Schulz.** 1994. The refined structures of a stabilized mutant and of wild-type pyruvate oxidase from *Lactobacillus plantarum*. *J. Mol. Biol.* **237**:315-35.
20. **Okamura, H., M. Denawa, R. Ohniwa, and K. Takeyasu.** 2003. P-type ATPase superfamily: evidence for critical roles for kingdom evolution. *Ann. N. Y. Acad. Sci.* **986**:219-23.

21. **Passardi, F., G. Theiler, M. Zamocky, C. Cosio, N. Rouhier, F. Teixera, M. Margis-Pinheiro, V. Ioannidis, C. Penel, L. Falquet, and C. Dunand.** 2007. PeroxiBase: the peroxidase database. *Phytochemistry* **68**:1605-11.
22. **Perdigon, G., S. Alvarez, M. E. Nader de Macias, and A. A. Pesce de Ruiz Holgado.** 1988. [Adjuvant activity of lactic bacteria: perspectives for its use in oral vaccines]. *Rev. Argent. Microbiol.* **20**:141-6.
23. **Pouwels, P. H., R. J. Leer, and W. J. Boersma.** 1996. The potential of *Lactobacillus* as a carrier for oral immunization: development and preliminary characterization of vector systems for targeted delivery of antigens. *J. Biotechnol.* **44**:183-92.
24. **Razeto, A., S. Kochhar, H. Hottinger, M. Dauter, K. S. Wilson, and V. S. Lamzin.** 2002. Domain closure, substrate specificity and catalysis of D-lactate dehydrogenase from *Lactobacillus bulgaricus*. *J. Mol. Biol.* **318**:109-19.
25. **Robinson, K., L. M. Chamberlain, K. M. Schofield, J. M. Wells, and R. W. Le Page.** 1997. Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nat. Biotechnol.* **15**:653-7.
26. **Saier, M. H., Jr., J. T. Beatty, A. Goffeau, K. T. Harley, W. H. Heijne, S. C. Huang, D. L. Jack, P. S. Jahn, K. Lew, J. Liu, S. S. Pao, I. T. Paulsen, T. T. Tseng, and P. S. Virk.** 1999. The major facilitator superfamily. *J. Mol. Microbiol. Biotechnol.* **1**:257-79.
27. **Steidler, L.** 2002. In situ delivery of cytokines by genetically engineered *Lactococcus lactis*. *Antonie Van Leeuwenhoek* **82**:323-31.
28. **Steidler, L., and P. Rottiers.** 2006. Therapeutic drug delivery by genetically modified *Lactococcus lactis*. *Ann. N. Y. Acad. Sci.* **1072**:176-86.
29. **Steinkraus, K. H.** 1983. Lactic acid fermentation in the production of foods from vegetables, cereals and legumes. *Antonie Van Leeuwenhoek* **49**:337-48.
30. **Suzuki, M., and T. Muranaka.** 2007. Molecular genetics of plant sterol backbone synthesis. *Lipids* **42**:47-54.
31. **Tanaka, H., Doesburg, K., Iwasaki, T., Mierau, I.** 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. *J. Dairy Sci.* **82**:2530-2535.
32. **Tannock, G. W., M. P. Dashkevich, and S. D. Feighner.** 1989. Lactobacilli and bile salt hydrolase in the murine intestinal tract. *Appl. Environ. Microbiol.* **55**:1848-51.
33. **Tannock, G. W., A. Tagerman, A. Van Schaik, and M. A. McConnell.** 1994. Deconjugation of bile acids by lactobacilli in the mouse small bowel. *Appl. Environ. Microbiol.* **60**:3419-20.
34. **Taupin, D., and D. K. Podolsky.** 2003. Trefoil factors: initiators of mucosal healing. *Nat. Rev. Mol. Cell. Biol.* **4**:721-32.
35. **Troost, F. J., R. J. Brummer, G. R. Haenen, A. Bast, R. I. van Haaften, C. T. Evelo, and W. H. Saris.** 2006. Gene expression in human small intestinal mucosa in vivo is mediated by iron-induced oxidative stress. *Physiol. Genomics* **25**:242-9.
36. **Uchikoba, H., S. Fushinobu, T. Wakagi, M. Konno, H. Taguchi, and H. Matsuzawa.** 2002. Crystal structure of non-allosteric L-lactate dehydrogenase from *Lactobacillus pentosus* at 2.3 Å resolution: specific interactions at subunit interfaces. *Proteins* **46**:206-14.
37. **Valle, F., P. Balbas, E. Merino, and F. Bolivar.** 1991. The role of penicillin amidases in nature and in industry. *Trends Biochem. Sci.* **16**:36-40.
38. **Van der Meer, R., J. W. Welberg, F. Kuipers, J. H. Kleibeuker, N. H. Mulder, D. S. Termont, R. J. Vonk, H. T. De Vries, and E. G. De Vries.** 1990. Effects of supplemental dietary calcium on the intestinal association of calcium, phosphate, and bile acids. *Gastroenterology* **99**:1653-9.

General discussion and concluding remarks

39. **Vincken, J. P., L. Heng, A. de Groot, and H. Gruppen.** 2007. Saponins, classification and occurrence in the plant kingdom. *Phytochemistry* **68**:275-97.
40. **Wallace, R. J.** 2004. Antimicrobial properties of plant secondary metabolites. *Proc. Nutr. Soc.* **63**:621-9.

Nederlandse samenvatting.

In de lever van zoogdieren worden galzouten gesynthetiseerd vanuit cholesterol en geconjugeerd met taurine of glycine. Na uitscheiding in de darm kunnen geconjugeerde galzouten gedeconjugeerd worden door leden van de endogene microbiota die het enzym galzout hydrolase produceren. Galzout hydrolase lijkt een belangrijke rol te spelen in zowel de darmfysiologie van de gastheer als in bacteriële overleving en persistentie in de darm; met name voor lactobacillen wordt gesuggereerd dat zij van belang zijn voor *in vivo* galzout hydrolyse in de dunne darm. In dit proefschrift is een functionele analyse van galzout hydrolase in Gram-positieve bacteriën, en in bijzonder van het model organisme *Lactobacillus plantarum* WCFS1 uitgevoerd. Dieper onderzoek naar de annotatie van galzout hydrolase genen in Gram-positieve bacteriën, gebruik makend van een combinatie van *in silico* methoden, leidde tot de re-annotatie van acht galzout hydrolase superfamilieleden in verschillende lactobacillen. Verder leverden deze analyses een robuuste methodologie voor de accurate annotatie van deze enzym superfamilie. *L. plantarum* WCFS1 was eerder voorspeld vier galzout hydrolase genen te bevatten (*bsh1*, *bsh2*, *bsh3* en *bsh4*), maar volgens onze *in silico* analyses zijn drie van deze genen mogelijk verwant aan penicilline acylases. Om de functionaliteit van elk van deze galzouthydrolase genen te onderzoeken was het nodig om meervoudige isogene galzouthydrolase-deletiestammen te maken. Voor de constructie van meervoudige selectiemarker-vrije deletiestammen is een Cre-*lox*-gebaseerde toolbox ontworpen en gebruikt in *L. plantarum* WCFS1. Gebruik makend van heterologe overexpressie en meervoudige galzouthydrolase-deletiestammen van *L. plantarum* WCFS1 werd aangetoond dat Bsh1 de belangrijkste galzout hydrolase is in deze stam. Bsh1 lijkt ook belangrijk te zijn voor de tolerantie van glycodeoxycholinezuur. Hoewel deze experimenten de voorspelling dat *bsh2*, *bsh3* en *bsh4* niet voor ware galzouthydrolases coderen valideerden, kon de *in vivo* functionaliteit van Bsh2, Bsh3 en Bsh3 niet volledig achterhaald worden. Bsh2, Bsh3 en Bsh4 lijken voor enzymen met acylase activiteit te coderen die mogelijk penicilline-achtige chemicaliën als voorkeurssubstraat gebruiken. Verder werd de invloed van galzouthydrolyserende probiotica op de fysiologie van de gastheer bestudeerd door gebruik te maken van twee manieren om galzouthydrolase af te leveren in de dunne darm; aflevering van galzout hydrolase activiteit door levende *L. plantarum* werd vergeleken met aflevering door galzout hydrolase/wei eiwit/arabische gom micro-encapsulaten in een *in vitro* model. De micro-encapsulaten gaven een uitstekende bescherming van het enzym onder maagcondities. Echter, onder pancreatinedruk tijdens darmcondities werd het Bsh enzym ge proteolyseerd. *L. plantarum* bleek in staat om zowel maag- als darmcondities te weerstaan, maar het niveau van enzymaflevering is relatief laag vergeleken met de capaciteit van micro-encapsulaten. Verder werd de invloed van aflevering van galzout hydrolase

activiteit door levende *L. plantarum* en wei eiwit/arabische gom micro-encapsulaten op de gastheer *in vivo* in een rat model bestudeerd. Er werd echter geen effect van de aflevering van galzout hydrolase op de galzout compositie of mucine excretie in de darm gevonden. Deze resultaten zouden kunnen betekenen dat de fysiologische relevantie en grootte van galzout hydrolase activiteit van probiotica in de dunne darm beperkt is.

Nawoord.

En dan is het tijd voor het stukje dat iedereen in ieder geval leest! Als ik zo terugkijk, heb ik echt veel plezier gehad in m'n AIO-periode. Niet alleen door het onderwerp, maar ook door alle mensen die daarbij betrokken waren.

Ik had dit proefschrift nooit kunnen maken als ik niet ontzettend geholpen was door Willem en Michiel. Willem, dank je voor je scherpe en constructieve opmerkingen, je was altijd een paar stappen voor me uit. Michiel, aan jou heb ik zoveel te danken! Niet alleen ben je een fantastisch persoon, je bent ook een ontzettend goede wetenschapper. Je hebt me ontzettend veel bijgebracht en ik heb onze samenwerking altijd als heel prettig ervaren.

En de mensen van NIZO: wat zijn jullie een stel leuke mensen, en ik ben blij dat ik een aantal van jullie toch blijf zien nu ik al een poos van NIZO weg ben. Mocht je je naam nergens kunnen vinden: sorry en dan maak ik wel een keer competente plantarums voor je ☺.

Allereerst Roger (sorry, dat wordt dus geen competente plantarums voor jou), dank je dat je paranimf wil zijn. Zelfs als je Tony's stropdas aan gaat doen. Je bent een hele goeie op het lab (en zeker ook daarbuiten), en ik denk dat het niet nodig zal zijn dat we samen op de vrachtwagen gaan. De mensen uit de denktank: Mariela (hoe is het met Mathilde?), Marc (binnenkort met Heidi), Arno (witte broek stond 'm goed), bedankt! Wat was het leuk met jullie, ik heb me altijd erg thuisgevoeld in de denktank. Peter, dank je dat je me op weg hebt geholpen als student. En dat je me geleerd hebt hoe je zo hoog mogelijk schuim kan krijgen met droogijs, en hoe je je RNA-gel zo lang mogelijk op de zuurkast kan laten plakken. En Gabriele, ik heb nog nooit iemand zo volhardend zien kolonie-PCR-en. Het was super om je op het lab te hebben, en je bent een ontzettend mooi mens.

Marke, Iris, Anne, Ingrid, Eddy, Tanja, Saskia, Bert, Marjo, Patrick, Filipe, Marieke, Sander, Michiel W., Roland, Bas, Douwe, Stijn, Rob, Sander, Vesela, Eline, Ronnie, Herwig, Johan, Frank, Matthé, Igor, Marleen: bedankt! Jacqueline, ik mis je ook hoor, moet nu voor iedereen de media maken. En Roelie, Jan, Kees, Guido, bedankt voor de hulp bij de HPLC. Roelof, Denise, Sandra, jullie hebben me echt onzettend geholpen met de dierproeven. Natuurlijk bedankt aan m'n studenten: Stefan, Ronald en Paula. Ik heb veel geleerd van jullie te begeleiden.

Verder natuurlijk nog de mensen uit C-007 die nog niet genoemd zijn: Cindy, Bart, Maaike, Maria, Sonja, Freddy, Andrea, Carien, Erwin. Bedankt! Er was altijd een prettige sfeer binnen onze groep.

En natuurlijk mijn familie en vrienden. Jullie betekenen heel veel voor me. Bedankt dat jullie m'n eeuwige gezeur over m'n boekje hebben willen aanhoren. Ingrid, Vasco, Maaïke, Remco, het is altijd zo goed om jullie te zien. Sandra, we kennen elkaar al aardig wat jaartjes inmiddels! Johan, Janneke, Richard, ik hou heel veel van jullie. Patrick, je bent niet meer weg te denken uit onze familie (en ik zou je ook niet graag meer missen). Connie, m'n supertweelingzussie en ook paranimf, je bent zo speciaal voor me. En pap, mam, bedankt voor jullie onvoorwaardelijke liefde en steun. Waar zou ik zijn zonder jullie.

Jolanda.

Curriculum vitae.

Johanna Maria Lambert (Jolanda) werd geboren op 18 april 1979 te Dirksland. Na de basisschool 'De Achtsprong' te Achthuizen volgde zij de VWO-opleiding aan de middelbare school 'Prins Maurits' te Middelharnis. Na het behalen van haar diploma begon zij in 1997 aan de studie Moleculaire Wetenschappen aan Wageningen Universiteit, een studie die haar interesse in moleculaire en microbiologie wekte. Tijdens deze studie deed zij onderzoek aan apomixis in *Arabidopsis thaliana* bij de vakgroep Moleculaire Biologie van Wageningen Universiteit onder leiding van Dr. Jenny Russinova en Prof. Sacco de Vries. Verder onderzocht zij het gebruik van het *alr* gen als een genetisch hulpmiddel in melkzuurbacteriën bij NIZO food research te Ede onder leiding van Dr. Peter Bron en Prof. Michiel Kleerebezem. Na het afronden van deze studie in eind 2001 werd haar de mogelijkheid geboden om voor het toenmalige Wageningen Centre for Food Sciences (heden Top Institute Food and Nutrition) een kort onderzoek naar het *ctsR* gen in *Lactobacillus plantarum* te doen, gevolgd door een promotieonderzoek naar de invloed van de productie van galzout hydrolase door de melkzuurbacterie *Lactobacillus plantarum* op de genexpressie van de epitheelcellen in de darm van de gastheer onder leiding van Prof. Michiel Kleerebezem en Prof. Willem de Vos. Het onderzoek werd gedaan onder de vlag van de leerstoelgroep Microbiologie van Wageningen Universiteit en maakte deel uit van het onderzoeksprogramma 'Microbial Functionality and Food Safety' van Wageningen Centre for Food Sciences, en had als titel 'Gastrointestinal host-microbe interactions and functional microbiomics: Directed host response modulations'. Het onderzoek werd uitgevoerd op NIZO food research te Ede en werd in 2007 afgerond. Op dit moment is zij werkzaam bij AM-Pharma te Bunnik.

Publications and patents.

Publications.

Use of the *alr* gene as a food-grade selection marker in lactic acid bacteria, Bron, P.A., Benchimol, M.G., Lambert, J., Palumbo, E., Deghorain, M., Delcour, J., De Vos, W.M., Kleerebezem, M., Hols, P. Applied and Environmental Microbiology. 2002, 68(11):5663-70.

Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. Pretzer, G., Snel, J., Molenaar, D., Wiersma, A., Bron, P.A., Lambert, J., de Vos, W.M., Van der Meer, R., Smits, M.A., Kleerebezem, M. Journal of Bacteriology. 2005, 187(17):6128-36.

Analysis of lactic acid tolerance in *Lactobacillus plantarum* derivatives mutated in lactic acid-stimulation associated functions. Pieterse, B., Lambert, J.M., Van der Werf, M.J., Kleerebezem, M. Ph.D.thesis, Chapter 5, Wageningen University. 2006.

Use of the SSLP-based method for detection of rare apomictic events in a sexual *AtSERK1* transgenic *Arabidopsis* population, Kantama, L., Lambert, J.M., Hu, H., De Jong, H., De Vries, S.C., Russinova, E. Journal of Sexual Plant Reproduction. 2006, 19(2):73-82.

Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. Lambert, J.M., Bongers, R.S., Kleerebezem, M. Applied and Environmental Microbiology. 2007, 73(4):1126-35.

Improved annotation of conjugated bile acid hydrolase superfamily members in Gram-positive bacteria. Lambert, J.M., Siezen, R.J., De Vos, W.M., Kleerebezem, M. Manuscript submitted.

Functional analysis of conjugated bile acid hydrolase family-members in *Lactobacillus plantarum* WCFS1. Lambert, J.M., Bongers, R.S., De Vos, W.M., Kleerebezem, M. Manuscript submitted.

In vitro analysis of Bsh enzyme protection against enteric conditions by whey protein-gum arabic microencapsulation. Lambert, J.M., Weinbreck, F., Kleerebezem, M. Manuscript submitted.

Patents.

Probiotic and compound derived thereof exhibiting immunomodulatory ability.
Konstantinov, S., Lambert, J.M., Van der Meer, C.M., De Vos, W.M.,
Kleerebezem, M. 2007.

VLAG graduate school activities.

Discipline specific activities.

BIT1- bioinformatics course (2003, VLAG).
Genetics and physiology of food-associated microorganisms (2004, VLAG)
Ecophysiology of the GI-tract (2003, VLAG)
System biology: principles of ~omics data analysis (2005, NUGO)

WCFS Food Summit (2003)
ALW Genetica meetings (2002-2006)
Lactic Acid bacteria 8 (2005, oral poster presentation)
Darmendag (2003)
Experimental evolution workshop (2004)

Working at animal facility WUR (2005)

General courses.

Techniques for writing and presenting a scientific paper (2003, PHLO)
Mondeling presenteren (2005, Copla)
WCFS intranet course, part of maintenance intranet WCFS

Optional.

Preparation PhD research proposal
Participation at and presentations of project and department meetings
Participation and presentations at WCFS We-Days (2002-2005)
WCFS PhD trips (2002-2003)
Literature study programme Microbiology WUR

