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FEMS Microbiology Reviews 29 (2005) 625–651

FEMS MICROBIOLOGY Reviews

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The interaction between bacteria and bile

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Received 19 May 2004; received in revised form 30 August 2004; accepted 14 September 2004

First published online 2 October 2004

Abstract

Commensal and pathogenic microorganisms must resist the deleterious actions of bile in order to survive in the human gastrointestinal tract. Herein we review the current knowledge on the mechanisms by which Gram-positive and Gram-negative bacteria contend with bile stress. We describe the antimicrobial actions of bile, assess the variations in bile tolerance between bacterial genera and examine the interplay between bile stress and other stresses. The molecular mechanisms underlying bile tolerance are investigated and the relationship between bile and virulence is examined. Finally, the potential benefits of bile research are briefly discussed.
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Keywords: Bile salt hydrolase; Gastrointestinal persistence; Probiotic; Stress; Virulence

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doi:10.1016/j.femsre.2004.09.003

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1. Introduction

To survive in the human gastrointestinal tract micro-organisms must endure numerous environmental extremes. Variations in pH, low oxygen levels, nutrient limitation and elevated osmolarity all constitute potential impediments to survival [1]. Given that the liver secretes as much as a litre of bile into the intestinal tract each day, exposure to bile also represents a serious challenge. Bile is a digestive secretion that plays a major role in the emulsification and solubilization of lipids. It has the ability to affect the phospholipids and proteins of cell membranes and disrupt cellular homeostasis. Therefore, the ability of pathogens and commensals to tolerate bile is likely to be important for their survival and subsequent colonization of the gastrointestinal tract.

This review begins with a brief overview of bile production and secretion and follows with a description of the antimicrobial actions of bile, analysis of variations in bile tolerance between various bacterial genera and investigation of the molecular mechanisms underlying tolerance. The relationship between bile and virulence is also examined. Finally, the potential benefits of bile research are briefly discussed.

2. Physiology of bile production and flow

2.1. Bile composition and functions

Bile is a yellow/green aqueous solution of organic and inorganic compounds (Table 1) whose major constituents include bile acids, cholesterol, phospholipids (mainly phosphatidylcholine) and the pigment biliverdin (bili = bile, verdi = green) [2–6]. Immunoglobulin A and mucus are secreted into bile to prevent bacterial growth and adhesion, and the presence of tocopherol may prevent oxidative damage to the biliary and small intestinal epithelium [4]. Many endogenous substances (endobiotics) may be secreted in bile and undergo enterohepatic cycling. These include lipovitamins (particularly the biologically active forms of vitamin D2), water-soluble vitamins (particularly vitamin B12, folic acid and pyrodoxine), all estrogenic steroids, progesterone, testosterone, corticosteroids and essential trace metals [2]. Many exogenous substances (xenobiotics) encountered by humans are also excreted into bile and undergo some degree of enterohepatic cycling. These include antimicrobial agents (e.g. clindamycin, rifampicin and erythromycin) and commonly used drugs (e.g. warfarin and morphine). Bile is generally isotonic with plasma with an osmolality of approximately 300 mOsm/kg that is primarily attributable to the osmotic activity of the inorganic ions [3,5].

Table 1
The major constituents and properties of human hepatic bile

Constituent/property	
Sodium (mmol/l)	145
Potassium (mmol/l)	4
Chloride (mmol/l)	90
Bile salts (mmol/l)	40
Cholesterol (mmol/l)	3
Phospholipids (mmol/l)	7
Dry weight (mg/ml)	20
Osmolality (mOsm/l)	280
pH	7.5–8.0

Adapted from [258].

The major function of bile in vivo is to act as a biological detergent which emulsifies and solubilizes fats. This also confers potent antimicrobial properties on bile and gives it an important role in the body's physicochemical defence system. Bile also functions as an excretory fluid by eliminating substances that cannot be efficiently excreted in urine because they are insoluble or protein bound, for example cholesterol which is derived from excess synthesis or the pigment bilirubin, the end product of heme metabolism, which is carried in blood attached to albumin proteins [4].

2.2. Bile synthesis, storage and secretion

Bile is synthesized in the pericentral hepatocytes of the liver and is secreted into thin channels called bile canaliculi. These canaliculi drain into bile ducts that merge to form hepatic ducts. Ultimately bile leaves the liver through the common hepatic duct that is joined by the cystic duct from the gallbladder to form the common bile duct (Fig. 1). Bile leaving this duct enters the duodenum at a junction regulated by a sphincter termed the sphincter of Oddi [4–6].

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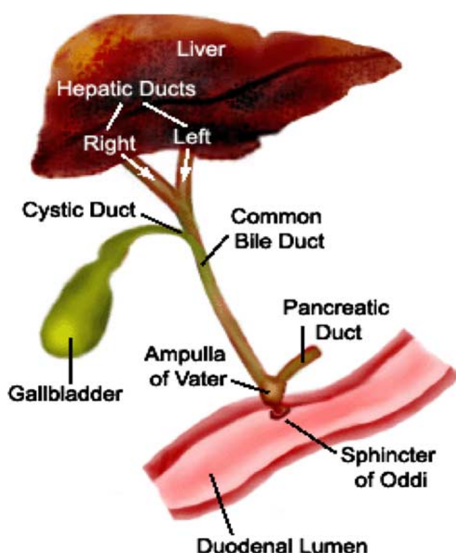

Fig. 1. Overview of the biliary system. Bile is synthesized in the liver, stored and concentrated in the gallbladder interdigestively and released into the duodenum upon diet intake by the host. (This figure is reproduced courtesy of the Faculty of Medicine Molson Informatics Project, Mc Gill University, Montreal, Canada.)

The gallbladder is a sac-like organ which, when full, expands to the size and shape of a small pear. Bile is diverted to the gallbladder interdigestively where it is concentrated approximately 5- to 10-fold. Water and electrolytes are removed and bile is acidified by Na^+/H^+ exchange [2,4,5]. The gallbladder is not essential for bile secretion but facilitates its storage in preparation for fat digestion. In fact, only half the hepatic bile enters the gallbladder for concentration and storage, the other half bypasses the gallbladder to enter the duodenum and undergoes continuous enterohepatic cycling. Many mammals and birds lack a gallbladder and cholecystectomy (gallbladder removal) does not result in maldigestion or malabsorption of fat [2,5].

When chyme from an ingested meal enters the small intestine, acid and partially digested fats stimulate secretion of secretin and cholecystokinin, respectively. These enteric hormones are important for the secretion and flow of bile. Secretin stimulates biliary duct cells to secrete bicarbonate and water to expand the volume of bile. Cholecystokinin (cholecysto = gallbladder, kinin = movement) stimulates contractions of the gallbladder and the common bile duct [5]. As a result, the gallbladder contracts, the sphincter of Oddi relaxes, and up to 80% of the gallbladder contents are discharged into the duodenum in an exponential fashion.

2.3. Bile acids

2.3.1. Biosynthesis and chemical structure

Bile acids constitute approximately 50% of the organic components of bile. They are synthesized in the liver from cholesterol by a multienzyme process. This biotransformation includes oxidative cleavage of the cholesterol side chain, resulting in the conversion of an isooctane moiety (side chain with eight carbons) into an isopentanoic acid moiety (side chain with five carbons) and addition of hydroxyl groups to the nucleus. Therefore, bile acids contain a perhydrocyclopentanophenanthrene steroid nucleus which consists of three six membered rings fused to a fourth five membered ring (Fig. 2(a)) [4,7]. Angular methyl groups at positions C17 and C19 make nineteen carbons in all for the steroid nucleus. The simplest C24 bile acid (with 3 and 7α hydroxyl groups and a C5 side chain) is termed chenodeoxycholic acid (abbreviated CDCA), and this bile acid is the building block of all other (C24) bile acids. A detailed description of all bile acids circulating in human bile is beyond the scope of this review, for more information see [7].

2.3.2. Bile acid conjugation

Preceding secretion, all bile acids are conjugated as N-acyl amidates (peptide linkage) with either glycine (glycoconjugated) or taurine (tauroconjugated) (Fig. 2(b)). Bile acids amidated with other amino acids, e.g. leucine or lysine, are rapidly hydrolysed by pancreatic carboxypeptidases [8]. The ratio of glycoconjugates to tauroconjugates in human bile is usually 3:1. However, this ratio can be as high as 9:1 in rural African women and as low as 0.1:1 in taurine-fed subjects [9,10]. Diets rich in taurine-containing foods such as meat and seafood will increase tauroconjugation [9]. Amidation has important effects on the solubility of bile acids since in the unconjugated form bile acids are only sparingly soluble at physiological pH. Conjugation lowers the pK_a of the terminal acidic group thereby allowing them to be freely soluble over a wide range of ionic strengths, calcium concentrations and pH values [11,12]. Taurine and glycine conjugates are often called bile salts by virtue of the fact that they are completely ionised at physiological pH, whereas the free (unconjugated) bile acids are not (the terms bile acids and bile salts are generally synonymous and are used interchangeably in this review). In addition to increasing solubility, amidation decreases passive absorption of bile acids in the biliary tract and small intestine [11]. The end result is to promote a high intraluminal concentration of bile that promotes excretion of lipids such as cholesterol and is essential for facilitation of fat digestion and absorption.

Bile acid substituents are on the α side of the molecule, making it hydrophilic, while the β side is free of substituents and hydrophobic. Bile acids are therefore amphipathic and, like all amphipathic molecules, can self-associate in water to form polymolecular aggregates called micelles (Fig. 2(c)) above a certain concentration termed the critical micellar concentration (CMC)

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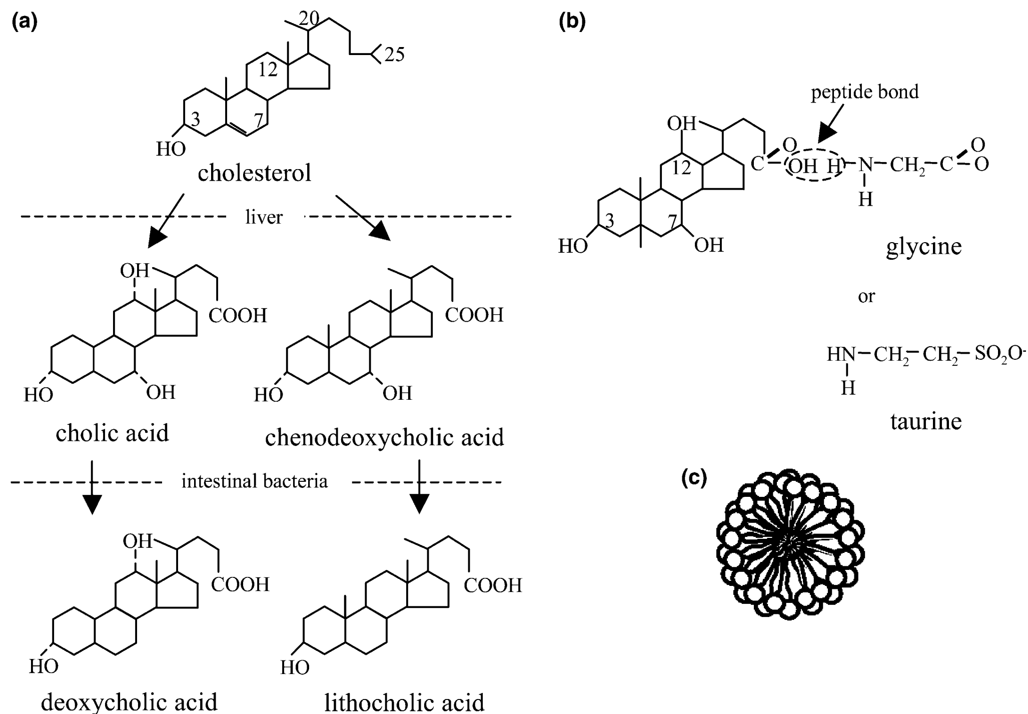

Fig. 2. (a) Chemical structure of the major bile acids of human bile. Primary bile acids (cholic acid and chenodeoxycholic acid) are synthesized in the liver from cholesterol; these can be modified by bacterial enzymes in the intestine to form secondary bile acids (deoxycholic acid and lithocholic acid). Other bile acids are present in human bile in trace proportions but the bile acids shown compose >95% of all biliary bile acids. Redrawn from [2]. (b) All bile acids are conjugated with either glycine or taurine before secretion. The carboxyl group of the bile acid and the amino group of the amino acid are linked by an amide bond (peptide linkage). (c) Bile acids are amphipathic and can self-associate to form polymolecular aggregates termed micelles. These micelles can solubilize other lipids in the form of mixed micelles.

[3,7,13]. Each micelle contains 4–50 molecules depending on bile acid structure. These micelles can, in turn, solubilize other lipids in the form of mixed micelles. The formation of mixed micelles of bile acids with phospholipids in bile lowers the monomeric activity of bile acids and prevents their destroying the apical membrane of biliary epithelial cells [4]. Bile acids below their CMC are surface active, binding to air–water and lipid–water interfaces [7].

2.3.3. Enterohepatic circulation

Bile acids are efficiently conserved under normal conditions. Conjugated and unconjugated bile acids are absorbed by passive diffusion along the entire gut and by active transport in the distal ileum [2]. Reabsorbed bile acids enter the portal bloodstream and are taken up rapidly by hepatocytes, reconjugated and resecreted into bile [14]. This whole process is termed enterohepatic circulation. Therefore, most of the bile acids transported by hepatocytes are "old" bile acids that were previously synthesized and secreted into bile. Intestinal absorption of the bile acid pool is about 95% efficient, so that faecal loss of bile acids is in the range of 0.3–0.6 g per day. This faecal loss is compensated by an equal daily synthesis of bile acids by the liver, and thus the size of the bile salt pool is maintained.

2.3.4. Bacterial alterations of bile acids

During passage through the caecum and colon, conjugated bile acids can be transformed by the indigenous bacterial flora. The three main alterations include deconjugation, 7α-dehydroxylation and 7α-dehydrogenation. Deconjugation results in cleavage of the amino acid side chain liberating free/unconjugated bile acids [15–18]. Dehydroxylation (removal of OH group) occurs at the 7 position of the nucleus and is therefore termed 7α-dehydroxylation. This reaction converts cholic acid to deoxycholic acid and chenodeoxycholic acid to lithocholic acid [19–21]. 7α-dehydrogenation converts chenodeoxycholic acid to 7-oxolithocholic acid, which can in turn be epimerised by bacterial enzymes to urodeoxycholic acid [22,23]. Some of the enzymes involved in these modifications will be discussed later in Section 6.2.

Therefore, there are two mechanisms and anatomical sites of bile acid biosynthesis. The first is de novo synthesis of so-called primary bile acids from cholesterol in the liver, the second is the production of secondary bile acids due to modification of primary bile acids by bacterial enzymes in the intestine.

2.3.5. Functions and dysfunctions of bile acids

Bile acids play an essential role in digestion by emulsifying and solubilizing fats. The bile acids secreted into

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bile form mixed micelles with phosphatidylcholine and cholesterol. When bile enters the small intestine, phosphatidylcholine is hydrolysed and absorbed and cholesterol precipitates from solution enhancing its elimination. The amphipathic nature of bile acids allows them to have detergent action on particles of dietary fat, which causes fat globules to break down or be emulsified into minute, microscopic droplets [4,5,7]. Emulsification greatly increases the surface area of fat, making it available for digestion by lipases, which cannot access the inside of fat droplets. Bile acids also function as "lipid carriers" in that they can solubilize lipids by forming micelles and thus allow their transport in an aqueous environment, which is critical for the absorption of fats and fat-soluble vitamins [7]. In addition, as stated previously, the ability of bile acids to act as detergents also allows them to interact with bacterial membrane lipids thereby conferring potent antimicrobial properties on bile.

The importance of maintaining the correct levels of bile acids is illustrated by inborn or acquired defects in bile acid synthesis or transport that result in abnormally high intracellular concentrations of bile acids. These conditions can induce necrosis and apoptosis, resulting in hepatocyte damage and death [24,25]. High intraluminal (extracellular) concentrations of bile acids induce secretion of electrolytes and water, manifested clinically as diarrhoea [4]. On the other hand, decreased bile acid intestinal concentrations, which may result from malnourishment or obstructions in enterohepatic circulation or intestinal absorption, leads to defective micellar solubilization of dietary lipids, which contributes to lipid malabsorption [26,27]. Decreased concentrations of bile acids in bile may also result in bile being supersaturated with cholesterol that may lead to the formation of gallstones [6,28]. These crystalline structures are formed by concretion or accretion of cholesterol monohydrate plus an admixture of calcium salts, bile acids, bile pigments, proteins, fatty acids and phospholipids. Finally, some of the secondary bile salts generated by microorganisms are potentially toxic and/or mutagenic [29–31]. It is suggested that they can disturb the normal microbiota of the gut leading to diarrhoea, mucosal inflammation or activation of harmful drugs and carcinogens in the intestinal contents [32].

3. Antimicrobial actions of bile

3.1. Membrane damage

That bile primarily exerts its effects on cell membranes was convincingly confirmed in experiments with erythrocytes. Addition of bile results in haemolysis and as erythrocytes do not possess organelles and lack specific mechanisms for the uptake and metabolism of bile salts, lysis can only be attributed to membrane-damaging effects [24,33–35]. The results of many other investigations indirectly suggest this mode of action. Environmental or physiological stimuli capable of altering membrane characteristics such as acid adaptation, increased osmolarity or entry into stationary phase render cells more resistant to the deleterious effects of bile [36–44]. In addition, the majority of loci disrupted in bile-sensitive mutants are associated directly or indirectly with the maintenance of membrane integrity (see Section 6). Finally, electron microscopy has shown that cells become shrunken and empty after exposure to bile [44,45] and enzyme assays have confirmed leakage of intracellular material [46,47], both implying that bile alters membrane integrity/permeability.

Several factors determine the exact outcome of bile action on cell membranes. Firstly, the concentration of bile is of major importance. Bile salts at high concentrations can rapidly dissolve membrane lipids and cause dissociation of integral membrane proteins [48,49]. This nearly instantaneous solubilization results in the leakage of cell contents and cell death. Low/submicellar concentrations of bile may disrupt membrane integrity through more subtle effects on membrane permeability and fluidity, including altered activity of critical membrane-bound enzymes and increased transmembrane flux of divalent cations [46,47,49,50]. Low levels of bile have also been shown to affect the physical chemical properties of cell surfaces, including hydrophobicity and zeta potential [51,52].

The type and structure of bile imposing the stress is another important factor. Binding of bile acids to membrane lipids correlates with their hydrophobicity [53,54]. As conjugated bile acids are strong acids, they are usually fully ionised at physiological pH values and remain in the outer hemileaflet of the bilayer unless a transport system is available. Unconjugated bile acids will flip-flop passively across the lipid bilayer and enter the cell. The rate of flip-flop depends on the number of hydroxy groups; dihydroxy bile acids flip rapidly, whereas trihydroxy bile acids flip much more slowly [55]. Bovine bile, which contains trihydroxyconjugated bile salts, is less inhibitory than porcine bile, which contains dihydroxyconjugated bile salts [56]. However, although bovine bile (oxgall) is commonly chosen to assess the in vitro bile tolerance of bacterial strains porcine bile is more similar to human bile with respect to bile salt/cholesterol, phospholipid/cholesterol and glycine to taurine ratios [57,58].

Finally, membrane architecture and composition play a key role in bile resistance. Alteration of membrane characteristics such as charge, hydrophobicity and lipid fluidity can have significant consequences. Conformational and structural damage of cell membrane lipopolysaccharides caused by freezing has been shown to increase the susceptibility of *Escherichia coli* 0157:H7

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cells to bile salts [59]. Carbon dioxide also interacts with cell membranes making them more permeable to bile. King et al. [60] observed that growing *Listeria monocytogenes* under CO_2 significantly affects the ability of cells to survive exposure to bile salts. Stationary phase cells grown under 40% CO_2:60% N_2 or 100% CO_2 were highly susceptible to bile salts, whereas cells grown under air or 100% N_2 were recovered following exposure to normally lethal concentrations of bile. Changes in fatty acid composition also affect bile tolerance. Fernandez Murga et al. [61] demonstrated that *Lactobacillus acidophilus* cells grown at 25 °C were more resistant to bile than cells grown at 37 °C. The authors concluded that fatty acid changes that occurred upon temperature downshift (more polyunsaturated (C18:2) and saturated (C16:0) fatty acids) contributed to enhancing the lipid membrane stability. Kimoto et al. [62] showed that culturing lactococcal strains with Tween 80 (polyoxyethylene sorbitan monooleate) produced strain-specific variations in fatty acid composition and enhanced bile tolerance. However, a relationship between fatty acid pattern and bile tolerance was not observed. Finally, bile tolerant mutants of *Lactobacillus acidophilus* isolated by Chou and Weimer [63] had altered cell wall fatty acid profiles amongst other differences.

3.2. Other effects

In addition to affecting membrane characteristics bile can have numerous other effects on bacterial cells including disturbing macromolecule stability. Bile acids have been shown to induce secondary structure formation in RNA, and also to induce DNA damage and activate enzymes involved in DNA repair in both bacterial and mammalian cells [25,44,64–67]. The detergent actions of bile may also alter the conformation of proteins resulting in their misfolding or denaturation. Indeed, the molecular chaperones DnaK and GroESL are induced by bile [38,39,44,68–70]. Bile may cause oxidative stress through the generation of oxygen free radicals [67,71,72]. Three of the eleven bile stress proteins identified by Leverrier et al. [44] in *Propionibacterium freudenreichii* play roles in oxidative damage remediation (cysteine synthase, oxidoreductase and superoxide dismutase) and Bernstein et al. [64] demonstrated that promoters that were most consistently induced by bile salts in *E. coli* included those of genes that are also responsive to oxidative stress (e.g. *micF* and *osmY*). The intracellular dissociation of bile salts may impose a low pH stress on cells and the movement of ions may have osmotic effects [16]. As bile can chelate calcium and iron [73,74] the presence of bile inside a cell may result in low intracellular calcium and iron concentrations.

It is evident that bile represents a plethora of challenges to a bacterial cell. The multifaceted nature of the stress it imposes is reflected in the diverse functions of genes that play a role in resistance (see Section 6.1).

4. Bile tolerance of various bacteria

4.1. Gram-negative bacteria

Little information is available on the bile tolerance of Gram-negative bacteria, but it is believed that they are inherently more resistant to bile than Gram-positive bacteria and bile salts are often used in their selective enrichment (e.g. Mac Conkey agar, *Salmonella*-*Shigella* agar, violet red bile agar and bile esculin agar) [75]. Van Velkinburgh and Gunn [76] determined that the minimal inhibitory concentrations (MICs) of oxbile (oxgall) for stationary phase cells of *Salmonella typhimurium* and *Salmonella typhi* were 18% and 12%, respectively, and minimal bactericidal concentrations (MBCs) were >60% for *S. typhimurium* and 18% for *S. typhi*. *Salmonellae* have the ability to colonize the gallbladder where bile concentrations are extremely high. This colonization is thought to be responsible for the chronic asymptomatic carrier state that occurs in 3–5% of infected people [77]. *E. coli* is also considered to be very bile resistant and is commonly isolated from the gallbladder and bile of animals and humans [78–82]. Growth of *E. coli* has been observed in the small intestinal compartments of a gastrointestinal tract model in the presence of high concentrations of porcine bile extract whereas Gram-positive bacteria were rapidly inactivated [83]. Certain species of *Helicobacter*, including *H. bilis* and *H. hepaticus*, inhabit the intestine and invade the bile ducts and liver [84,85], while in contrast, *H. pylori* is sensitive to bile perhaps explaining why it has never been isolated from faecal samples or cholecystectomy specimens [86,87]. *Campylobacter* are also considered bile resistant and have been isolated from the gallbladder and directly from bile [88–93].

4.2. Gram-positive bacteria

4.2.1. Gram-positive probiotic commensals

In addition to resistance to low pH, adhesion to gut epithelial tissue and production of antimicrobial substances, resistance to bile toxicity is one of the criteria used to select probiotic strains that would potentially be capable of performing effectively in the gastrointestinal tract [94–99]. Therefore, to date, most of the work on bacterial bile tolerance has been performed by probiotic researchers. However, since bile resistance is only one of many criteria used to select strains, analyses are not in depth and usually simply compare the ability of a whole collection of strains to grow in the presence of a particular type of bile. The results of numerous studies of this type are available in the literature but they are

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difficult to objectively assess. Different experimental conditions (different types of bile, broth, etc.) make it impossible to directly compare all results. In addition, many data is interpreted subjectively and researchers tend to classify the bile tolerance of strains arbitrarily. Therefore, as it would be meaningless to cite all available data, certain probiotic studies are chosen to highlight two recurring observations.

Firstly, Gram-positive bacteria seem to be more sensitive to the deleterious effects of bile than Gram-negative bacteria. The MICs of oxgall for the 19 *Bifidobacteria* strains examined by Margolles et al. [100] ranged from 0.125% to 2%. Of the 13 sporeforming lactic acid producing bacteria examined by Hyronimus et al. [101] (different strains of *Sporolactobacillus*, *Bacillus laevolacticus*, *Bacillus racemilacticus* and *Bacillus coagulans*) only 5 strains were tolerant to bile (oxgall) concentrations over 0.3%. Most (31 of 47) *Lactobacillus* strains examined by Jacobsen et al. [102] did not replicate in broth supplemented with 0.3% oxgall and growth of the remaining strains was delayed. The five *L. acidophilus* strains examined by Noh and Gilliland [47] all grew more slowly in broth containing 0.3% oxgall and several reports highlight the bile sensitivity of *Lactococcus* strains [62,103–105].

A second observation made by several investigators is that bile tolerance is a strain-specific trait and tolerances of species cannot be generalized. Numerous studies portray the extreme variability in resistance that can be found within a species or genus. Chateau et al. [106] tested the effect of bile salts on a collection of 38 strains of *Lactobacillus* (mainly *L. rhamnosus*) isolated from a probiotic bacterial consortium and observed a heterogeneity of sensitivity. Of 22 *L. rhamnosus* strains, 3 strains were classified as resistant, 5 as tolerant, 3 as having a low tolerance and 11 as sensitive. Similar variability was observed by Jacobsen et al. [102] when they compared the bile tolerance of 47 strains of *Lactobacillus* spp. (included *L. plantarum*, *L. fermentum*, *L. rhamnosus*, *L. reuteri*, *L. acidophilus*, *L. crispatus*, *L. paracasei*, *L. casei* and *L. johnsonni*) and numerous investigators examining collections of *L. acidophilus* strains [107–110]. Zarate et al. [50] classified *Propionibacterium* strains into tolerant and non-tolerant groups and observed that *P. freudenreichii* was present in both groups. Ibrahim and Bezkorovainy [111] examined survival of *Bifidobacterium* strains in bile and reported that *B. infantis* had the best survival rates and *B. longum* had the lowest. In contrast, Clark and Martin [112] reported that *B. longum* was much more tolerant of bile than *B. infantis*.

4.2.2. Gram-positive pathogens

Detailed information on the bile tolerance of Gram-positive pathogens is limited. *Listeria monocytogenes* cholecystitis (colonization of the gallbladder) has been reported, which suggests an inherent tolerance of very high levels of bile [113–116]. In our laboratory, we have demonstrated that *L. monocytogenes* strain LO28 is able to tolerate concentrations of bovine, porcine and human bile well in excess of those encountered in vivo. Confluent growth was observed on agar plates supplemented with 15% oxgall, 15% bovine bile, 2% porcine bile or 15% human bile (the highest concentrations tested) and cells could survive and even grow in broth supplemented with 30% oxgall [36]. A recent study by Olier et al. [117] investigated the bile tolerance of fifty human asymptomatic carriage, clinical, food and environmental isolates of *L. monocytogenes* and demonstrated that all could grow in broth supplemented with 5% porcine bile salts again suggesting that *L. monocytogenes* strains are inherently bile resistant. However, both our study and that of Olier et al. revealed that, similar to probiotic strains, listerial bile tolerance is also subject to strain variation and all strains do not exhibit equally high levels of tolerance. Both studies reveal that *L. monocytogenes* strain EGDe which was recently sequenced by the European *Listeria* Genome Consortium is significantly more bile-sensitive than strain LO28 [36,117]. In addition, Olier et al. did not find a correlation between bile tolerance and the origin of the strains. In contrast to both of these studies King et al. [60] observed that exponential phase cells of four *L. monocytogenes* isolates examined were extremely sensitive to bile and were not detectable after 2 min in 0.3% oxbile. Discrepancies between studies may be explained by experimental differences concerning growth conditions such as pH of broth used. We have shown that toxicity of glycoconjugated bile salts is strongly pH dependent with their toxicity increasing as pH decreases [36].

Flahaut and co-workers [38,39] demonstrated a rapid killing of *Enterococcus faecalis* by a mix of unconjugated bile salts (sodium cholate–sodium deoxycholate [1:1]). Incubation with 0.3% led to a 1000-fold decrease in survival after 15 s [39]. However, as *E. faecalis* is one of the predominant microbial species isolated from biliary drain devices used in biliary surgery [118] and has been isolated from bile [80,81], it is possible that the particular mix of bile used by Flahaut et al. does not truly reflect in vivo situations, where bile salts are primarily conjugated and less toxic and the bacterium is more tolerant of bile than suggested by their in vitro experiments.

Whilst little precise data is available concerning bile tolerance of *Clostridium* spp., *Clostridium perfringens* has been isolated from human and animal bile suggesting an inherent bile tolerance in this pathogen [79,119].

4.3. Factors affecting bile tolerance

It must be noted that the tolerance of strains in bile broth systems may not truly reflect their ability to

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tolerate bile in vivo. Like other physiological stresses, it is difficult to simulate exact in vivo conditions in a laboratory setting and all parameters that can affect survival are not taken into account. Conditions encountered in the external environment or in the host prior entry to the small intestine will determine the effects of bile on a strain. Exposure to various pHs, temperatures and growth atmospheres may either "harden" bacteria to the affects of bile or alternatively increase their susceptibility (see Section 5). Bile acid levels in the intestine are not constant and levels are relatively low until ingestion of a fatty meal. Pre-exposure of bacteria to these low levels may increase their tolerance to high levels (see Section 5). The presence of food in the intestine may also affect survival as bacteria may not be exposed to bile in certain microenvironments created by the food matrix or food constituents may even bind bile acids and prevent them from exerting toxicity. Gänzle et al. [83] observed that *Lactobacillus curvatus* was rapidly inactivated by bile (porcine bile extract) in the small intestinal compartments of a gastrointestinal tract model. However, addition of meat exerted a protective effect and resulted in increased delivery of cells to the intestinal compartment. Shimakawa et al. [120] observed that the inhibition of *Bifidobacterium breve* Yakult by bile was partially alleviated by addition of soy protein, which has been shown to bind bile acids and aggregate them [121]. Encapsulation of probiotic strains increases their bile tolerance, e.g. Hou et al. [122] demonstrated that viability of *Lactobacillus delbrueckii* ssp. *bulgaricus* was elevated by approximately four-log-units after encapsulation within artificial sesame oil emulsions. Finally, the in vivo antibacterial activity of bile may be lower than observed in broth systems as bile salts complexed in micelles with phospholipids may not be free to interact with bacterial cells.

5. Bile adaptation and cross-adaptation

Food-borne pathogens, such as *Listeria*, may encounter many environmental insults during food production, preparation and storage and following consumption including low pH, volatile fatty acids, low oxygen levels and bile [1]. Pre-exposure to sublethal levels of a given stress has been shown to allow cells to adapt and protects them against subsequent exposure to normally lethal levels of the same stress [123,124]. Pre-exposure to one stress may also confer protection against other stresses, a phenomenon termed cross-adaptation [124–127]. This is not altogether surprising given that many stresses have similar effects on cellular physiology and may well cause induction of the same set of stress proteins. It is becoming increasingly obvious that many stress management response systems overlap and are interconnected.

The most detailed studies which exist on bile adaptation and cross-adaptation were performed on *E. faecalis* by Flahaut and co-workers [37–40]. As mentioned in Section 4, the bile salts used in their experiments (a mix [1:1] of sodium salts of two unconjugated bile acids – cholic acid and deoxycholic acid) resulted in extremely rapid killing. There was nearly a 4-log-unit reduction in cell numbers after exposure to 0.3% wt/vol for 15 s [39]. However, adaptation to extremely low levels of bile (0.08%) for a period of as little as 5 s (flash adaptation) provided substantial protection against challenge with normally lethal levels [39]. Addition of chloramphenicol during the adaptation period did not prevent development of acquired tolerance, suggesting that adaptation to this mix of bile salts was independent of protein synthesis [39]. When these exact experiments were carried out on *L. monocytogenes* in our laboratory, results mirrored those of Flahaut et al. in that 5 s was a sufficient adaptation time and adaptation was independent of protein synthesis [36]. As detergents form mixed micelles with lipids resulting in rapid solubilization and disaggregation [128] it is possible that low levels of bile rapidly intercalate with membrane lipids rendering the subsequent mixed membranes resistant to further detergent effects. Using the same mix of bile salts (cholic acid and deoxycholic acid) Leverrier et al. [44,129] showed that *P. freudenreichii* was also capable of adapting to bile, however the adaptation characteristics appeared to be different from *E. faecalis*. No significant tolerance was observed for a 5 min pre-treatment and the optimal induction period was 4 h. Electron microscopy revealed that non-adapted cells appeared shrunken and empty when exposed to lethal levels of bile suggesting leakage of intracellular material. Besides a decrease in cell length, adaptation to bile did not result in notable changes in morphology. When exposed to lethal levels of bile these adapted cells did not show any obvious modifications, suggesting that adaptation prevents cell disruption possibly by altering cell wall properties. Schmidt and Zink [70] found that both log and stationary cells of *Bifidobacterium adolescentis* could adapt to bile. Pre-conditioning log cells for 30 min to 0.1% bile (oxgall) resulted in a 300-fold and 21-fold protection against 0.3% and 0.4% bile salts, respectively. The only report of bile adaptation in a Gram-negative bacterium was for *S. typhimurium* by Van Velkinburgh and Gunn [76] in which pre-treatment of exponential phase cells with low levels of bile (1–3%) did not affect resistance, however pre-treatment with 15% bile (crude oxbile extract) resulted in increased survival when challenged with 24% (400% vs. 100% for non-adapted). Two-dimensional (2D) gel analysis showed that several additional proteins were induced by 15% bile when compared to 3%, suggesting they might mediate adaptation. Exposing stationary phase cells to 15% bile did not increase their resistance when subsequently challenged with 24% bile.

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Adapting *E. faecalis* cells to heat (50 °C), sodium chloride (6.5%), sodium dodecyl sulphate (SDS) (0.017%) or alkaline conditions (pH 10.5) all conferred an increased ability to survive otherwise lethal levels of bile, while pre-treatment with bile salts induced significant thermotolerance [37–40]. Acid pre-treatment did not cross-protect against bile salts although bile salt adaptation did lead to an increased sensitivity to acid challenge [38]. 2D gel electrophoresis experiments showed that 45 proteins were induced by 0.08% bile (the level used in adaptation experiments) [38,39]. Comparing autoradiograms with those obtained after exposure to sublethal levels of other stresses revealed significant overlap between proteins. Further physiological analyses showed proteins induced during adaptation to alkali were necessary for the acquisition of bile salt tolerance. However, the observed cross-tolerance by salt adaptation could not be attributed to overlaps in proteins as the same level of cross-protection to bile was observed in cells that were adapted to salt with or without de novo protein synthesis [40]. Collectively, experiments carried out by this group suggest that although overlapping proteins may play a role in the cross-protection observed between other stresses and bile, other mechanisms also exist. These most likely involve alterations in membrane characteristics; in fact, all of the sublethal stresses that provided cross-protection are known to induce membrane changes [127,130–133].

Carbon starvation, heat adaptation (42 °C) or SDS pre-treatment (0.06%) conferred significantly increased protection of *Priopionibacterium* towards bile salt challenge. Cold (4 °C) or osmotic (0.3 M NaCl) pre-treatments had no effect on tolerance while acid pre-treatment (pH 5) sensitised cells [44]. Exposing exponential phase *L. monocytogenes* cells to acid (pH 5.5), heat (42 °C), salt (5% NaCl) or SDS (0.01%) increased bile tolerance [36]. Log phase cells of long-term starved cells of *L. lactis* exhibited higher resistance against bile salt stress compared to the parent (25% vs. 0.6% when exposed to 0.1% sodium cholic acid–deoxycholic acid) [41,103]. *Lactobacillus casei* cells grown under hypersaline conditions (1 M NaCl) were more sensitive to the action of bile salts (0.05–0.35% wt/vol oxgall) than cells grown in media without added salt [134]. Acid-adapted and alkali-adapted cells of *Vibrio parahaemolyticus* exhibited increased tolerance to deoxycholate when compared to non-adapted cells [42,43].

It is obvious that stresses encountered in food-processing environments or in vivo could potentially influence bacterial bile tolerance either directly by changing expression of particular genes or indirectly by altering membrane characteristics such as permeability, fluidity or charge. In addition, bacteria capable of adapting to low levels of bile that would be present in the intestine during the fasting state may adapt and increase their ability to survive the high levels of bile released upon dietary intake by the host.

6. Genetics of bacterial bile tolerance

A wealth of information exists on the molecular mechanisms employed by bacteria to sense and resist physiologically relevant stresses, for example the low pH stress of the stomach or the elevated osmolality of the gastrointestinal tract. In contrast, the genetics of bile resistance is poorly understood, particularly in Gram-positive organisms. The modes of action of bile on bacterial cells have been outlined in Section 3 of this review. Given the complicated nature of this stress, the ability of an organism to tolerate bile will presumably require a wide array of proteins, including many which govern cell envelope architecture or maintenance of intracellular homeostasis. Proteins that take up or extrude bile, or enzymes that modify and transform bile salts are also likely to play important roles in bile resistance. This section will include the information available on the effects of bile on bacterial protein expression and the genes that have been shown to contribute to bile stress survival (summarised in Tables 2 and 3). Bacterial bile salt deconjugation will be discussed in detail. Finally, modes of sensing bile and regulating the responses it triggers will be proposed.

6.1. Proteins induced by bile/genes involved in bile tolerance

6.1.1. Gram-negative bacteria

6.1.1.1. Salmonella. In common with other Gram-negative bacteria, the *Salmonella* outer membrane is an excellent barrier to membrane active agents such as bile. The lipopolysaccharide (LPS) plays a major role in resistance and loss of the O-antigen, which creates a "rough" colony phenotype, results in increased sensitivity [135,136]. It has been shown that charge modifications to the LPS molecule (aminoarabinose addition to lipid A and phosphoethanolamine modifications to the core and lipid A regions) do not affect bile resistance therefore electrostatic interactions between bile and the *Salmonella* LPS do not seem to be important [76,137]. Multidrug efflux pumps can remove bile that gets through the outer membrane, e.g. the *S. typhimurium* AcrAB pump is absolutely required for bile resistance [138–140]. Many Gram-negative transport systems require a Tol protein to function as their outer membrane pore protein [141] and mutations in *tol* genes also affect resistance to bile. Three of the fifteen bile-sensitive *S. typhimurium* mutants isolated from a MudJ transposon mutant bank by Prouty et al. [136] were located in the *tolQRA* region. Mutations in *tol* genes destabilize the membrane allowing greater access for bile salts entry.

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Table 2
Loci disrupted in bile-sensitive mutants and the functions/putative functions of gene products

Genes disrupted in bile-sensitive mutants	Function/putative function of gene products	Reference(s)
Gram-negative bacteria		
Salmonella enterica		
acrAB	Efflux pump	[138–140]
tolQRA, *tolC*	Efflux pump function	[136]
phoPQ	Regulatory genes	[76]
marAB	Regulatory genes	[144]
dam	DNA adenine methylase	[143]
wecD, *wecA*	Biosynthesis and assembly of enterobacterial common antigen	[142]
Escherichia coli		
acrAB	Efflux pump	[146]
emrAB	Efflux pump	[146]
mdtABCD	Efflux pump	[148]
ompF	Outer membrane porin	[146]
ompC	Outer membrane porin	[146]
hupAB	DNA-binding protein, controls DNA supercoiling	[259]
rfa	Lipopolysaccharide core biosynthesis	[147]
Vibrio cholerae		
waaF	heptosyl II transferase	[150]
wavB	1-4-β-glycosyltransferase	[150]
vceAB	Efflux pump	[151]
tolC	Efflux pump function	[152]
ompU	Porin	[153,154]
galU	UDP-glucose-pyrophosphorylase	[149]
galE	UDP-glucose-4-epimerase	[149]
Campylobacter jejuni		
cmeAB	Efflux pump	[156,157]
Gram-positive bacteria		
Enterococcus faecalis		
sbcC homolog	Exonuclease	[160]
mutS homolog	DNA mismatch repair	[160]
nifJ homolog	Pyruvate flavodoxin oxidoreductase	[160]
yvaG homolog	Probable 3-oxoacyl-[acyl-carrier-protein] reductase	[160]
dgt homolog	dGTP triphosphohydrolase	[160]
Bacillus halodurans regulator homolog	Transcriptional regulator	[160]
sagA homolog	Cell wall lytic activity	[160]
Streptomyces coelicolor CAC16441 homolog	Putative esterase	[160]
Streptococcus thermophilus orf1091 homolog	Unknown	[160]
Enterococcus faecalis orf8	Unknown	[160]
Listeria monocytogenes		
lmo0516	Membrane biogenesis	[163]
lmo1451	Membrane biogenesis	[163]
lmo0448	Transmembrane transporter	[163]
btlA	Transmembrane transporter	[163]
lmo0605	Transmembrane transporter	[163]
lmo1416	Transmembrane transporter	[163]
lmo1442	Transmembrane transporter	[163]
lmo1408	Transcriptional regulator	[163]
zurR	Transcriptional regulator	[163]
lmo1450	RNA helicase	[163]
pflB	Pyruvate formate lyase	[163]
bsh	Bile salt hydrolase	[162,163]
pva	Penicillin amidase	[163]
sigB	stress sigma factor	[163]
btlB	Unknown	[163]
lmo1432	Unknown	[163]
Lactobacillus amylovorus		
bsh	Bile salt hydrolase	[56]

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Table 3
Promoters[a], proteins[b] or open reading frames (ORFs)[c] induced by bile and their functions/putative functions

ORFs/promoters/proteins induced by bile	Function/putative function	Reference(s)
Gram-negative bacteria		
Escherichia coli		
micF	Small regulatory RNA gene[a]	[64]
osmY	Unknown, stress responsive[a]	[64]
dinD	Unknown, DNA damage inducible[a]	[64]
Gram-positive bacteria		
Enterococcus faecalis		
DnaK	Molecular chaperone[b]	[69,160]
GroEL	Molecular chaperone[b]	[69,160]
Ohr	Organic hydroperoxide resistance gene[b]	[158]
Gsp62, 63, 64	General stress proteins[b]	[69,160]
Propionibacterium freudenreichii		
DnaK	Molecular chaperone[b]	[44,129]
Hsp20	Heat shock protein, molecular chaperone[b]	[44]
ClpB	ATP-binding chain of an ATP-dependent protease[b]	[44,129]
AlgU homolog	Putative alternative sigma factor[b]	[44]
RecR homolog	Recombinase (DNA repair)[b]	[44,129]
SodA	Superoxide dismutase[b]	[44,129]
BCCP	Biotin-containing carboxyl carrier protein[b]	[44,129]
CysK homolog	Cysteine synthase[b]	[129]
ORF0001	2 component system regulatory protein, signal transducer[b]	[44]
ORF0002	NADPH-dependent aldo- or keto-oxidoreductase[b]	[44]
HtrII homolog	Sensory rhodopsin II transducer[b]	[44]
OppD homolog	Oligopeptide transport ATP-binding protein[b]	[129]
TufA homolog	Elongation factor Tu[b]	[129]
MutB homolog	Methylmalonyl-CoA mutase large subunit[b]	[129]
AspA homolog	Aspartate ammonia-lyase[b]	[129]
G6PD homolog	Glucose-6-phosphate 1-dehydrogenase[b]	[129]
ATPG homolog	ATP synthase gamma chain[b]	[129]
CssR homolog	Transcriptional regulatory protein[b]	[129]
MurF homolog	Probable UDP-N-acetlymuramoyl-tripeptide-D-alanyl-D-alanine ligase[b]	[129]
HemH homolog	Ferrochetalase[b]	[129]
Listeria monocytogenes		
groESL	Molecular chaperones[c]	[68]
Lactobacillus plantarum		
lp_0085	Cation efflux protein[c]	[165]
lp_0237	Integral membrane protein[c]	[165]
lp_1435	Integral membrane protein[c]	[165]
lp_2564	Integral membrane protein[c]	[165]
lp_3155	Cell surface protein[c]	[165]
lp_3160	Multidrug transport protein[c]	[165]
lp_3175	Integral membrane protein[c]	[165]
lp_3626	Sugar transport protein[c]	[165]
lp_1158	Lysozyme[c]	[165]
lp_3153	Muramidase[c]	[165]
lp_3154	Muramidase[c]	[165]
lp_0082	Oxidoreductase[c]	[165]
lp_0858	Redox protein[c]	[165]
lp_3145	Oxidoreductase[c]	[165]
lp_3158	Oxidoreductase[c]	[165]
lp_3489	Oxidoreductase[c]	[165]
lp_1157	Transcription regulator[c]	[165]
lp_2484	Transcription regulator[c]	[165]
lp_3344	Transcription regulator[c]	[165]
lp_3345	Negative regulator of proteolysis[c]	[165]
lp_3488	Galactose operon repressor[c]	[165]
lp_0774	Autoinducer production protein[c]	[165]
lp_0775	Arginosuccinate synthase[c]	[165]
lp_2835	2-haloacid dehalogenase[c]	[165]
lp_3159	Bile acid 7α-dehydratase[c]	[165]

(*continued on next page*)

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Table 3 (*continued*)

ORFs/promoters/proteins induced by bile	Function/putative function	Reference(s)
lp_1459	Unknown[c]	[165]
lp_0240	Unknown[c]	[165]
lp_2230	Unknown[c]	[165]
lp_3330	Unknown[c]	[165]
lp_3343	Unknown[c]	[165]
lp_3415	Unknown[c]	[165]

Loci regulated by the two-component system PhoPQ are also required for bile resistance in *S. typhimurium*. Van Velkinburgh and Gunn [76] demonstrated that mutants lacking PhoPQ were killed at significantly lower bile (crude oxbile extract) concentrations than wild-type strains, while strains with constitutively active PhoP were able to survive prolonged incubation with bile at concentrations of >60%. Reporter gene fusions indicated that the PhoPQ regulon does not sense and respond to bile. This suggests that the PhoPQ regulon and the ''bile regulon'' contain overlapping genes, which are responsible for the bile resistance phenotype. These loci have not yet been identified.

Mutations in *wecD* and *wecA* genes both of which are involved in the biosynthesis and assembly of enterobacterial common antigen (ECA) render *S. typhimurium* 40-fold more sensitive to the bile salt deoxycholate than the wild-type [142]. In addition, *S. typhimurium* DNA adenine methylase (DAM) mutants exhibit enhanced sensitivity to bile salts [143]. This enzyme may be important in repairing DNA damage induced by exposure to bile.

The *Salmonella marAB* operon, a regulatory locus that controls multiple antibiotic resistance [144], has recently shown to be activated in the presence of bile and this response is concentration-dependent (2.9-fold increase in transcriptional activity in 1% sodium cholate up to 5.3-fold induction in 5% bile) [140]. The bile salt deoxycholate is alone able to activate transcription, while there was no response in the presence of other bile salts tested (taurocholate, glycocholate and glycochenodeoxycholate) or a non-ionic detergent (Triton X-100).

SDS–PAGE analyses by Van Velkinburgh and Gunn [76] revealed that several proteins were affected both positively and negatively by bile (3% crude oxbile extract) and deoxycholate (1%) in *S. typhimurium* and *S. typhi*. Bile resulted in 15 observable changes (14 increased and 1 decreased) and deoxycholate resulted in 14 changes (7 increased and 7 decreased) in *S.* typhimurium. A smaller number of alterations were observed in *S. typhi* (2 for bile (1 increased and 1 decreased) and 6 for deoxycholate (2 increased and 4 decreased)). Minimal overlap was observed in the proteins affected for each serovar suggesting that the regulatory factors or the targeted genes differ. Recent DNA microarrays by the same laboratory showed 230 genes to be >3-fold affected when *S. typhimurium* cells were exposed to 3% bile [145]. Of these 230 genes, 101 were activated in the presence of bile while 129 were repressed.

6.1.1.2. Escherichia coli*.* Similar to *Salmonella*, the LPS and efflux pumps play important roles in *E. coli* bile resistance. The majority of bile-sensitive mutants isolated from transposon Tn*phoA* banks by Thanassi et al. [146] were deep rough lipopolysaccharide mutants producing a hyperpermeable outer membrane. Several of the mutants with changes in their LPS structure isolated by Picken and Beacham [147] were bile-sensitive and many of the mutations mapped in or near the *rfa* locus. The AcrAB and EmrAB multidrug efflux systems have been shown to actively efflux bile salts (chenodeoxycholic acid) [146]. Efflux of bile by an *acrAemrB* double mutant suggests the involvement of other unknown system(s) [146]. Overexpression of the multidrug resistance cluster *mdtABCD* leads to increased resistance to deoxycholate [148].

As bile acids can traverse the membrane through porins, it is not surprising that the nature of porins produced influences susceptibility to bile. A strain expressing the OmpF porin that produces a channel with a wider diameter is more susceptible to the inhibitory effect of deoxycholate than a strain which expresses the porin with the smaller diameter channel OmpC [146].

Bernstein et al. [64] examined the activation of 13 *E. coli* stress response genes by bile salts (glycocholate, ursodeoxycholate, chenodeoxycholate and deoxycholate). The most consistently activated promoters were those for genes that protect against DNA damage and oxidative stress (*micF* which regulates the outer membrane OmpF and is induced by oxidative stress; *osmY* a periplasmic protein that is osmotically inducible and is also induced by oxidative stress; and *dinD* which is induced by DNA damage as part of the SOS response).

6.1.1.3. Vibrio cholerae*.* Loci important in *V. cholerae* bile resistance include genes for LPS synthesis, efflux pumps, Tol proteins and porins. Mutants with the LPS core oligosaccharide biosynthesis genes, *waaF* (heptosyl II transferase), *wavB* (1-4-β-glycosyltransf-

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erase), *galU* (UDP-glucose-pyrophosphorylase) or *galE* (UDP-glucose-4-epimerase), affected grow poorly in bile [149,150]. Analysis of a mutant that lacks the VceAB efflux pump demonstrated a significant role for this system in tolerance of deoxycholate [151]. In addition, mutation of the *V. cholerae tolC* gene that encodes a homologue of the *E. coli* outer membrane protein, TolC, demonstrated a dramatically reduced growth rate at bile acid concentrations as low as 0.02% [152]. In detailed analyses of specific outer membrane proteins, ''porin swap'' experiments show that cells with only OmpU in the outer membrane are more resistant to deoxycholate than cells with only OmpT [153,154]. Furthermore, OmpU expression is stimulated by bile and is dependent upon ToxR the central regulator of virulence factor expression in *V. cholerae* [153]. However, virulence studies have ruled out a role for OmpU in colonisation of the infant mouse intestine [155].

6.1.1.4. Campylobacter jejuni. The CmeABC multidrug efflux pump is essential for growth of *C. jejuni* in bile salts-containing media. Lin et al. [156] demonstrated that inactivation of the pump confers exquisite sensitivity to various bile salts. The MICs of selected bile salts were decreased 4000-fold (chenodeoxycholic acid), 1000-fold (deoxycholic acid) and 64-fold (cholic acid and taurocholic acid), and when grown in sodium choleate (crude oxbile extract) a *cmeB* mutant was not detected after 3 h whereas the wildtype demonstrated normal growth over the 50-h period [156,157].

6.1.2. Gram-positive bacteria

6.1.2.1. Enterococcus faecalis. 2D-PAGE analysis in *E. faecalis* showed increased production of 45 proteins during bile salts treatment [38,39]. Seven of these proteins were induced by several stress conditions and were designated general stress proteins. Three were identified as the molecular chaperones DnaK and GroEL and Ohr an organic hydroperoxide resistance protein [69,158,159]. To identify genes involved in bile resistance a plasmid (pORI19) integration bank was screened for mutants sensitive to 0.2% wt/vol unconjugated bile salts (cholic acid–deoxycholic acid) [160]. Disrupted loci encoded homologues of proteins involved in DNA repair (MutS and SbcC), oxidative response (NifJ), transcription regulation (*Bacillus halodurans* BAB04138 homolog), dGTP hydrolysis (Dgt), membrane composition (YvaG) or cell wall synthesis (SagA and *Streptomyces* CAC16441 homolog).

6.1.2.2. Propionibacterium freudenreichii. Leverrier et al. [44,129] used a proteomic approach to analyse the response of *P. freudenreichii* to various sub-lethal stress conditions. 2D electrophoresis experiments revealed that at least 24 proteins were induced following exposure to bile salts (0.02% cholic acid–deoxycholic acid). Six of these proteins were common to all of the stresses examined (acid, heat and bile) and included the molecular chaperones DnaK and ClpB, and BCCP a biotin containing carboxyl carrier protein. Bile salts specific proteins included a putative two-component sensor kinase, a homolog of a sigma factor (AlgU) and proteins that play roles in DNA damage repair (RecR) or oxidative damage remediation (SodA, cysteine synthase and oxidoreductase) [44,129].

6.1.2.3. Listeria monocytogenes. Phan-Thanh and Gormon [161] examined protein expression in *L. monocytogenes* LO28 under a variety of stress conditions. Addition of 0.03% deoxycholate to media induced 13 newly synthesized proteins and up-regulated 18 proteins, none of which were identified. In support of previous findings in *E. faecalis*, induction of the *groESL* operon in response to bile has also been demonstrated in *L. monocytogenes* LO28 [68]. Dussurget et al. [162] utilised the genome sequence of *L. monocytogenes* EGDe to locate the *bsh* gene that encodes a bile salt hydrolase enzyme that degrades bile acids (see Section 6.2) and analysed the role of this locus in resistance to bile. It was observed that the MICs for porcine bile and purified bile salts are 2-fold lower than the wild-type when the gene is inactivated (0.08% and 0.15%, respectively). We have recently confirmed their data in *L. monocytogenes* LO28 and have demonstrated that another *bsh* homologue *lmo0447* (which we have designated *pva*), as well as a putative bile acid dehydratase *lmo0754* (which we have designated *btlB*) are essential for full bile tolerance [Begley et al., submitted for publication]. In addition, we have recently employed a transposon-based approach to identify and characterize loci that contribute to inherent bile tolerance. Disrupted loci in bile-sensitive mutants included genes putatively involved in membrane biogenesis (*lmo0516* and *lmo1451*), membrane transport (*btlA*, *lmo0448*, *lmo0605*, *lmo1416* and *lmo1442*), anaerobic metabolism (*pflB*), macromolecule stability (*lmo1450*) and transcriptional regulation (*lmo1408* and *zurR*) [36,163,164]. Physiological analyses revealed that growth of specific bile-sensitive mutants was significantly affected under a variety of other stress conditions (acid – pH 5.5, alkaline – pH 9, salt – 7% NaCl, ethanol – 5%, SDS – 0.05%) suggesting that loci contribute to general stress resistance [163,164, Begley et al., submitted for publication].

6.1.2.4. Lactobacilli. Recently, bile-inducible genes were identified in *Lactobacillus plantarum* WCFS1 by screening an *alr* (alanine racemase) complementation library [165]. Forty-six plasmid derivatives containing promoter elements conditionally activated by 0.1% porcine bile were isolated. The partial sequence of chromosomal inserts present in 41 of these clones was determined and they corresponded to 31 unique loci of the *L. plantarum*

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genome. Open reading frames (ORFs) were functionally categorized in groups involved in cell membrane function (lp_0085, lp_0237, lp_1435, lp_2564, lp_3155, lp_3160, lp_3175 and lp_3626), cell wall function (lp_1158, lp_3153 and lp_3154), redox reactions (lp_0082, lp_0858, lp_3145, lp_3158 and lp_3489), regulation (lp_1157, lp_2484, lp_3344, lp_3345 and lp_3488), and others (lp_0774, lp_0775, lp_2835 and lp_3159). The remaining 6 genes (lp_0240, lp_1459, lp_2230, lp_3330, lp_3343 and lp_3415) encode hypothetical proteins of unknown function. Quantitative reverse transcription PCR analyses of two of these loci; lp_0237 which encodes a putative integral membrane protein and lp_0775 which encodes arginosuccinate synthase, showed that their expression levels are induced (24- and 4-fold, respectively) in vitro in *L. plantarum* cells grown on MRS plates containing 0.1% porcine bile relative to control plates lacking bile [165]. In addition, expression of these loci was also increased in RNA extracted from the duodenum of mice infected with *L. plantarum* (13- and 29-fold, respectively) compared to expression levels of cells grown on MRS. Clone-based DNA microarrays were employed by the same laboratory to investigate the global transcriptional response of *L. plantarum* towards porcine bile [165]. Comparison of the differential transcript profiles obtained during growth on plates with and without 0.1% bile revealed 29 and 62 putative genes of which the expression was at least 2.5-fold up- or down-regulated by bile. Up-regulated genes included those that encode proteins involved in stress responses (e.g. lp_1253 which encodes glutathione reductase, and lp_3420 which encodes glutamate decarboxylase) or in cell envelope integrity and function (e.g. lp_2019/2020 which encode proteins involved in teichoic acid biosynthesis and lp_2364–2369 which encode an F1F0 ATPase). Six bile-repressed genes encode cell membrane located transporters. The authors suggest that bile stress-induced loss of membrane integrity can partially be compensated by the down-regulation of genes encoding non-essential membrane proteins.

Grill et al. [56] used a N-methyl-N^1-nitro-N-nitrosoguanidine (NTG) mutagenesis strategy to isolate a *Lactobacillus amylovorus bsh* (encodes a bile salt hydrolase enzyme; see Section 6.2.1) mutant. Growth rates of this mutant were significantly lower than the parent when grown in the presence of a glycoconjugated or a tauroconjugated bile salt (glycodeoxycholic acid and taurodeoxycholic acid, respectively).

6.2. Bacterial bile salt modifications

In humans, approximately 0.3–0.6 g of bile acids (approximately 5% of total) per day eludes epithelial absorption and may undergo extensive modifications by the indigenous intestinal microflora. Transformations include epimerisation (inversion of the stereochemistry of the hydroxyl groups at C-3, C-7 and C-12), deconjugation (removal of the amino acid side chain), oxidation (expulsion of H_2), reduction (insertion of H_2), hydroxylation (replacement of a hydrogen with a hydroxyl group) and dehydroxylation (replacement of a hydroxyl group with a hydrogen). Only deconjugation and 7-dehydroxylation are discussed in this review, the other transformations are reviewed by Bortolini et al. [22].

6.2.1. Bile salt deconjugation

6.2.1.1. Bile salt hydrolases. Bile salt hydrolases (BSHs) are enzymes (EC 3.5.1.24) that catalyze the hydrolysis of the amide bond between the C-24 position of the steroid moiety and the amino acid side chain of bile acids. BSHs belong to the choloylglycine hydrolase family of enzymes that also contains penicillin amidases (EC 3.5.1.11) and both have recently been classified as N-terminal nucleophilic (Ntn) hydrolases. Amino acids that are thought to play a role in catalysis (Cys-1, Asp-20, Tyr-82, Asn-175 and Arg-228) are conserved in both [166,167]. Penicillin amidases hydrolyse penicillin to yield 6-aminopenicillinic acid (6-APA), which is widely used in the industrial production of semi-synthetic antibiotics with a wide range of antimicrobial activities [168]. The microbial role of penicillin amidases has not yet been elucidated. It has been suggested that they may hydrolyse bile acids and that these enzymes and BSHs share substrates [15,167], but this has not yet been investigated. Alternatively, as they hydrolyse phenylacetylated compounds, penicillin amidases may have a role in the non-parasitic environment in generating carbon sources [168,169].

BSHs are generally intracellular enzymes that are oxygen insensitive, have a slightly acidic optimal pH (usually between pH 5 and 6), their activity is coupled to biomass production and they are not regulated by bile salts [15–18,170–179]. Exceptions to these generalizations include the extracellular BSH of *Clostridium perfringens* [180], the BSH of *L. acidophilus* which requires low oxidation–reduction potential [172] and the BSH of *L. johnsonni* strain 100–100 which is inducible by bile [181]. Indeed, BSH activity of *L. johnsonni* strain 100–100 increases 3–5-fold within 20 min after conjugated bile salts are added to stationary phase cells. This increase is due to induction of BSH by an uncharacterized extracellular factor [181]. Finally, BSH activity in *L. johnsonni* strain 100–100 and in *Bacteroides fragilis* only increases when organisms enter into stationary phase [18,177].

Hydrolysis of bile salts is mediated by various genera of the resident intestinal microflora such as *Clostridium* [173,182], *Bacteroides* [175,177], *Lactobacillus* [15,16,18,56,181,183–188], *Bifidobacterium* [17,178,189] and *Enterococcus* [190,191]. The recent identification of a BSH in *L. monocytogenes* by Dussurget et al. [162] is

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the first description of the enzyme in a pathogenic species that is not considered a member of the normal enteric flora. Interestingly, the authors note that the G + C content of the *bsh* gene is lower than neighbouring genes and suggest that it may have been acquired from low G + C content bacteria such as lactobacilli [162].

6.2.1.2. Function(s) of BSH. The ecological significance of microbial BSH activity is not yet fully understood, although three major hypotheses have been advanced. Firstly, it has been proposed that deconjugation may confer a nutritional advantage on hydrolytic strains as liberated amino acids could potentially be used as carbon, nitrogen and energy sources. Glycine may be metabolised to ammonia and carbon dioxide, and taurine to ammonia, carbon dioxide and sulphate, that could then be incorporated into bacterial metabolites. To support this theory, Van Eldere et al. [192] and Huijghebaert et al. [193] observed that certain strains of *Clostridia* utilize the released taurine as an electron acceptor. In addition, transcription of the *B. longum bsh* gene is coupled to a homolog of *glnE* that encodes a glutamine synthetase adenyltransferase that is part of the nitrogen regulation cascade [178]. However, other studies have shown that lactobacilli do not utilize the steroid moiety of bile salts as cellular precursors suggesting that this is not a universal function of BSHs [172,188].

Secondly, it has been proposed that BSHs facilitate incorporation of cholesterol or bile into bacterial membranes [194–196]. This incorporation may increase the tensile strength of the membranes [197] or may change their fluidity or charge in a way that could affect sensitivity to α-defensins and other host defense molecules [198,199]. Pridmore et al. [200] suggest that this adaptation could strongly select for commensals with BSHs, while disfavouring pathogens or other transients lacking BSHs.

Finally, it is possible that deconjugation of bile salts may be a detoxification mechanism and BSH enzymes may play a role in bile tolerance and consequently in survival in the gastrointestinal tract. Numerous investigators have refuted this hypothesis. Taranto et al. [201] did not find a relationship between the ability of 9 *E. faecium* strains to grow in bile (0.3% oxgall) and their ability to hydrolyse bile salts (glycocholic acid or taurocholic acid). Similar studies by Gopal et al. [108] (6 strains of *L. acidophilus* and 8 strains of *Bifidobacterium* spp.), Usman and Hosono [202] (28 strains of *L. gasseri*), Moser and Savage [203] (49 strains of numerous *Lactobacillus* spp.) and Ahn et al. [204] (5 *L. acidophilus* strains) did not correlate bile tolerance with BSH activity. However, studies by three independent groups using wild-type and *bsh* mutant pairs provide a link between bile salt hydrolysis and bile tolerance [16,56,162]. As previously mentioned the *Lactobacillus amylovorus* mutant with a partial decrease in BSH activity isolated by Grill et al. [56] displayed decreased growth rates in the presence of bile salts. Dussurget et al. [162] showed that deletion of *bsh* in *L. monocytogenes* decreased the MICs for both bile and bile salts. Indeed, we have recently independently verified their results [163, Begley et al., submitted for publication]. Finally, a *L. plantarum bsh* mutant created by De Smet et al. [16] showed a strong pH-dependent inhibition due to the presence of a glycoconjugated bile acid (glycodeoxycholic acid) but not a tauroconjugated bile acid (taurodeoxycholic acid). The authors suggest that, as the protonated (non-dissociated) form of bile salts are thought to exhibit toxicity through intracellular acidification in a manner similar to organic acids, BSH positive cells may protect themselves through the formation of the weaker unconjugated counterparts. This could help negate the drop in pH by recapturing and exporting the co-transported proton (Fig. 3). Taken together, these three studies with *bsh* mutants strongly suggest a role for BSH in bile tolerance, and particularly tolerance to glycoconjugated bile salts. It is possible that the contradictory studies may have used inappropriate experimental conditions; for example, using tauroconjugated bile acids to detect BSH activity even though the majority of BSHs show a preference for glycoconjugated bile acids. In addition, the bile tolerance of strains was often assayed by growth in low levels of bile where BSH activity may not be important.

The unconjugated bile acids resulting from bile salt hydrolysis have greater inhibitory effects on bacteria than

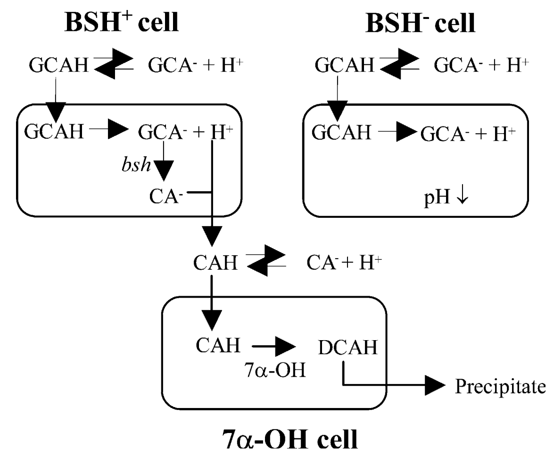

Fig. 3. Schematic representation of the hypothesis that bile salt hydrolysis is a detoxification mechanism. Bile salt hydrolases convert the protonated-conjugated bile salts that enter cells to their weaker unconjugated counterparts which may recapture co-transported protons thereby preventing the excessive expenditure of ATP to maintain pH homeostasis. A large energy burden may be put on BSH negative cells since they cannot form unconjugated bile salts that trap the protons. 7α-dehydroxylating bacteria may dehydroxylate unconjugated bile acids and the resulting molecules precipitate at moderately acidic pH (Adapted from 16,185]). *Abbreviations*: BSH – bile salt hydrolase, 7α-OH – 7α dehydroxylating, GCAH – glycocholic acid, CAH – cholic acid, DCAH – deoxycholic acid.

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conjugated bile acids in vitro. However, it is possible that they are precipitated at the low pHs in the intestine caused by the fermentation products of lactic acid bacteria. In fact, this localized precipitation phenomenon is the basis for the agar plate assay used to detect BSH activity [184]. De Smet et al. [16] observed that the toxic effect of unconjugated bile acids on *L. plantarum* could be partially alleviated by the addition of a metabolizable carbon source, and suggested that BSH active strains might be capable of detoxifying bile acids by an energy-dependent process. It has also been suggested that BSH positive strains may associate with 7α-dehydroxylating bacteria that would dehydroxylate unconjugated bile acids [185]. Dehydroxylated molecules have low solubility and precipitate at moderately acidic pH.

A role for BSH in colonization of the gastrointestinal tract was investigated in *L. monocytogenes* [162]. A *bsh* mutant demonstrated reduced bacterial faecal carriage after oral infection of guinea pigs. It was also observed that intestinal multiplication of the parent could be increased by supplying cells with an extra copy of the gene on a plasmid, and also that BSH activity was exclusive to pathogenic species of *Listeria*, further confirming the role of BSH in persistence of *L. monocytogenes* within the gastrointestinal tract. It is likely that future investigations will reveal a similar role for BSHs of other organisms. In fact, it has been noted that BSH activity is found primarily in organisms isolated from the gastrointestinal tracts of mammals (*Bifidobacterium* spp., *L. acidophilus*, *L. gasseri*, *L. johnsonni*, some strains of *L. plantarum*), while organisms isolated from environments from which bile salts are absent such as fermented milk preparations and vegetables (*L. lactis*, *L. delbruecki*, *L. helveticus*, *Strep. thermophilus*) do not exhibit BSH activity [186,203,205]. Interestingly, the complete genome sequences of intestinal probiotic bacteria have revealed that some strains may possess more than one *bsh* gene e.g. *L. johnsonii* NCC533 has three [200] and *L. plantarum* WCFS1 has four [206] perhaps highlighting the importance of BSH to intestinal survival.

6.2.1.3. Impact of microbial BSH activity on the host. Deconjugation has important consequences for the physicochemical properties of bile acids. As unconjugated bile acids are less efficient than conjugated molecules in the emulsification of dietary lipids and the formation of micelles, lipid digestion and absorption of fatty acids and monoglycerides could be impaired [207]. Microbial BSH activity has been related to growth depression in chickens [208,209] but not in mice [183]. Efficient enterohepatic recirculation of bile acids is partially dependent on their recognition in the conjugated form by active transport sites in the terminal ileum. Unconjugated bile acids bind with a lower affinity to the transport sites and thus may pass into the large intestine or caecum. This may result in enhanced faecal loss of bile salts that would increase the demand for cholesterol for de novo bile salt synthesis that may in turn lower serum cholesterol levels. In fact, a reduction in serum cholesterol levels has been demonstrated in pigs, minipigs, and germfree and conventional mice administered with BSH-active bacteria [207,210–214]. These cholesterol-lowering effects have received much attention from probiotic researchers as it offers potential as a "biological" approach to treating hypercholesterolaemia. However, according to some authors, BSH activity of probiotics would not be favourable as subsequent modification of unconjugated bile salts could generate toxic compounds, which could disturb the normal microbiota of the gut leading to diarrhoea, mucosal inflammation or activation of carcinogens in the intestinal contents [29,31,32,66,215].

6.2.2. 7α-dehydroxylation

Specific members of the genera *Eubacterium* and *Clostridium* are the only intestinal bacteria that have been shown to 7α-dehydroxylate (remove the OH group at the 7 position) the primary bile acids (cholic and chenodeoxycholic acid) into secondary bile acids (deoxycholic and lithocholic acid, respectively) [19–21,194, 216,217]. In this multistep transformation, primary bile acids are actively transported into the cell and conjugated to coenzyme A. The bile acid-coenzyme A conjugate is sequentially oxidized to a 3-oxo and then a 3-oxo-$\Delta^{4,6}$-intermediate that is sequentially reduced in three steps to the 7α-dehydroxylated bile acid that is released from the cell [20,21,216,217]. A bile acid inducible (*bai*) operon that contains at least nine open reading frames, encodes the enzymes required for the pathway [19–21,216,217]. The physiological role of 7α-dehydroxylation is unknown, however, it has been suggested that this pathway provides the bacterium with an ancillary electron receptor [20,217]. Bile acid 7α-dehydroxylation products are potentially toxic to humans, and increased levels of these in the bile acid pool have been associated with an increased risk of cholesterol gallstone formation and colon cancer [28,218–220].

6.3. Bile sensing and regulation of the bile stress response

The mechanisms employed by bacteria to sense and respond to bile are currently unknown but are likely to be similar to those used for other stress responses.

Bile may be sensed directly by two-component systems consisting of a membrane-associated histidine kinase and a cytoplasmic response regulator. The histidine kinase generally monitors an environmental parameter and when it senses a change (e.g. the presence of bile), it signals a response regulator which then instructs the cell to respond to the change, often via regulation of gene expression [221]. The direct sensing of bile may also involve transcriptional activators. Usually the binding

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of signal molecules (e.g. bile components) alters the conformation of these proteins and transcription of particular genes is then activated.

Cells may sense bile indirectly by responding to the consequences of exposure, e.g. the disruption of membrane integrity. Known bacterial membrane damage sensors include the *E. coli* Cpx and RcsCb systems, which sense alterations in the cell surface and induce the expression of genes involved in stress responses [222,223]. Bile-induced alterations in the states of macromolecules may also function as sensors and signal transducers. It is possible that exposure to bile may influence DNA supercoiling levels in a manner similar to other stresses (pH and osmolarity) where variations act as a sensor of external change and co-ordinate gene expression patterns [224–230]. In fact, many supercoiling-sensitive promoters are induced by bile in *E. coli* [64]. In addition to activating certain promoters, increasing or decreasing DNA supercoiling levels can have consequences for other topological perturbations of DNA, e.g. looping or bending, that may affect transcription [231]. It is also possible that accumulation of proteins damaged or denatured by bile may trigger induction of a general stress response.

Transcription of genes in response to bile is likely to be controlled by transcriptional regulators including sigma factors. Bile has been shown to upregulate the *Campylobacter jejuni* flagellar *flaA* σ^{28} promoter [232] and 2D electrophoresis experiments have revealed that the *Propionibacterium freudenreichii* sigma factor homolog AlgU is induced following exposure to bile [44,129]. Considering the large number of stress genes thought to be involved in bile resistance it is likely that the two well-characterized sigma factors SigmaB (σ^B) and RpoS (σ^S) that control stress-induced regulons in Gram-positive and Gram-negative bacteria, respectively [233–236] will have significant roles in bile stress responses. In fact, we have recently demonstrated that mutation of *sigB* impairs the ability of *L. monocytogenes* LO28 to tolerate bile and transcription of *bsh* whose product plays an important role in listerial bile tolerance has been shown to be regulated by SigmaB [163,237,238, Begley et al., submitted for publication].

7. Bile and pathogenesis

Bacteria continuously monitor environmental parameters during colonization or infection of the host, and subsequently express genes that assist in survival and repress those which are unnecessary. It is therefore not surprising that virulence factors are co-ordinately regulated by a variety of environmental signals such as acid, temperature and osmolarity [239]. Two well-characterized examples include the *Salmonella* virulence regulator PhoPQ which is responsive to acidic pH, Mg^{2+} concentrations and to carbon and nitrogen starvation [240], and PrfA, the principal virulence regulator in *L. monocytogenes*, which is regulated by temperature and carbohydrate source [241]. It is becoming increasingly obvious that enteric pathogens can also use bile as an environmental cue to establish location and influence the regulation of virulence genes. However, in addition to using bile to control established virulence determinants, it is likely that some of the gene products involved in bile tolerance will contribute to survival and colonization of the intestinal tract and thus, in their own right, function as virulence factors.

7.1. Gram-negative bacteria

7.1.1. Salmonellae

The role of bile in *Salmonellae* pathogenesis is well recognised. Transcription of *S. typhimurium* invasion gene regulators (e.g. *sirC* and *invF*) is repressed in the presence of bile which results in a marked decrease in transcription of *Salmonella* pathogenicity island 1 (SPI-1) genes involved in epithelial cell entry [145,242]. As a result, bacteria grown in the presence of bile are less able to invade epithelial cells than those grown without bile [242,243]. The bacterium therefore uses bile as an environmental signal to repress its invasive capacity in the intestinal lumen where bile concentrations are high, while invasion may then be initiated when bile concentrations have decreased, e.g. after transit of the mucus layer covering the epithelium in the distal ileum (Fig. 4(a)). In addition, *Salmonellae* show reduced expression of flagellar biosynthesis genes including *flhC*, *flgC* and *fliC*, and reduced motility in the presence of bile [145].

Bile has also been shown to promote the formation of *Salmonellae* (*S. typhi* and *S. typhimurium*) biofilm on human gallstones in vitro in a bile dependent manner [77]. If biofilm formation was to occur in vivo not only would it protect bacteria from the high levels of bile in the gallbladder but it would also limit the effectiveness of certain antibiotics. Biofilm formation on gallstones may therefore contribute to establishment of the chronic asymptomatic carrier state that occurs in 3–5% of infected people [77].

Inactivation of the *S. typhimurium* AcrB multidrug efflux pump that is involved in bile resistance reduces colonization of this organism in the intestinal tract of mice [138]. Furthermore, the *S. typhimurium* two-component system PhoPQ is absolutely required for both bile tolerance and virulence in mice [76,240].

Mutation of *S. typhimurium wecD* and *wecA* genes which are involved in biosynthesis and assembly of enterobacterial common antigen ECA results in strains that are highly attenuated in oral infections but only show a slight decrease in virulence during intraperitoneal infection. As mutation of these loci renders

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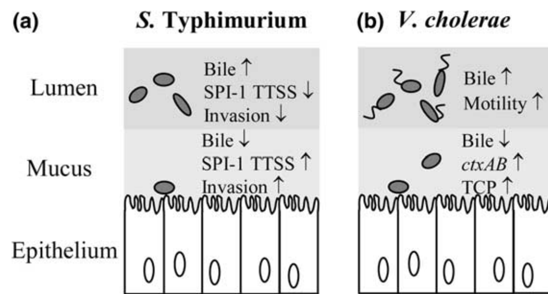

Fig. 4. (a) Model of the effect of bile on *Salmonella enterica* serovar *typhimurium* invasion. When the bacterium is within the intestinal lumen bile inhibits transcription of invasion gene regulators. This results in a decrease in SPI-1 genes involved in epithelial cell entry. After transit of the mucus layer where bile concentrations decrease, invasion genes are derepressed (Adapted from [242]). (b) *Vibrio cholerae* "motility first, virulence gene expression second" model. Bile present in the lumen results in decreased virulence gene expression (perhaps via inactivation of ToxT) and increased motility. When the bacterium swims into the mucus gel bile concentrations decrease, motility is blocked and virulence gene expression is induced (Adapted from [252]). *Abbreviations*: SPI-1-TSS – *Salmonella* pathogenicity island 1 genes, *ctxAB* – cholera toxin genes, TCP – toxin co-regulated pilus.

Salmonella bile-sensitive, Ramos-Morales et al. [142] therefore suggest that ECA contributes to *Salmonella* virulence by protecting the pathogen from bile salts.

7.1.2. Shigellae

Contrary to the negative effects of bile on *Salmonella* invasion, Pope et al. [244] showed that growth of *Shigella* spp. (*S. flexneri* and *S. dysenteriae*) in bile salts (deoxycholate or chenodeoxycholate) results in an increase in Ipa protein secretion and increased invasion of epithelial cells due to enhanced adherence. Growth in the presence of other structurally similar bile salts or detergents had little or no effect suggesting a specific interaction between certain bile salts and virulence gene expression in this organism.

7.1.3. Vibrio cholerae/parahaemolyticus

Bile acids play a key role in the pathogenicity of *Vibrio* species. Conjugated bile acids enhance production of *V. parahemolyticus* thermostable direct hemolysin (TDH), a virulence factor produced by strains most frequently associated with disease [245,246]. In addition, bile acids enhance the expression of other attributes associated with virulence such as Congo red binding ability, capsule production and adherence to epithelial cells [247]. Pace et al. [247] also observed a large and rapid decrease in intracellular calcium in *V. parahaemolyticus* cells treated with bile (oxgall). The authors suggest that as bile acids are calcium chelators, bile may trigger a low calcium response, which is known to regulate virulence genes in other pathogens [248,249].

In contrast to *V. parahaemolyticus*, it has been demonstrated that growth of *V. cholerae* in the presence of bile results in decreased virulence gene expression [250,251]. Addition of bile to the growth medium dramatically reduced transcription of the *ctxAB* genes encoding cholera toxin (CT) and CT production was decreased by more than 97% [250]. It was also noted that addition of bile resulted in increased motility. This may assist bacteria to penetrate the mucosal layer and gain access to the underlying epithelial cells [252]. A study by Schuhmacher and Klose [251] demonstrated that bile inhibits the activity of the transcription factor ToxT that activates numerous promoters required for colonization and disease in the host including those for CT and the toxin co-regulated pilus (TCP) genes. The authors propose that once bacteria swim to a location where bile concentrations are low, inhibition of ToxT activity will be relieved resulting in expression of genes required for effective colonization (Fig. 4(b)).

Mutation of the *V. cholerae waaF*, *warB* (two LPS core oligosaccharide genes) or *tolC* (encodes the outer membrane pore protein of efflux pumps) genes significantly impacts the organisms ability to grow in bile. Mutation of these loci also results in colonization defects in the infant mouse model of infection [150,152].

7.1.4. Campylobacter jejuni

Bile salts (sodium deoxycholate, cholate and chenodeoxycholate) affect invasiveness by stimulating the synthesis of *Campylobacter* invasion (Cia) proteins [253]. It was observed that synthesis was specific for bile as altering pH, calcium concentration, osmolarity or temperature did not induce their synthesis. One of these invasion proteins, CiaB has been shown to play a role in chicken cecal colonization [254].

Bile upregulates the *C. jejuni* flagellar *flaA* σ^{28} promoter [232]. An increase in FlaA synthesis and hence chemotaxis likely maximizes the likelihood of colonizing the mucus layer and pathogenesis. It has also been reported that bile (deoxycholate) stimulated the production of *Campylobacter* pili, which are known to be important virulence determinants in many pathogenic bacteria [255]. However, further investigations revealed that these appendages were not pili but were bacteria-independent morphological artefacts of the growth medium [256].

The CmeABC multidrug efflux pump is absolutely essential for successful colonization of *C. jejuni* in chickens, most likely by mediating resistance to bile salts [157]. In controlled experiments, the wild-type strain colonized chickens as early as day 2 postinoculation whereas the *cmeABC* mutants failed to colonize any of the inoculated chickens throughout the 20-day study [157].

7.2. Gram-positive bacteria

7.2.1. Enterococcus faecalis

It has been shown that growth of *E. faecalis* strains in bile (oxbile) alters the physicochemical surface proper-

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ties of the bacterium and results in increased invasion of biliary drain materials [52]. Since biomaterial centered infections with *E. faecalis* are initiated by bacterial adhesion it was therefore concluded that growth in bile would increase the likelihood of infection by this route.

7.2.2. *Listeria monocytogenes*

Recent in vivo bioluminescence imaging experiments performed by Hardy and co-workers [115] revealed that *L. monocytogenes* can replicate in the murine gallbladder and its replication there is extracellular and intraluminal. Strong signals were observed from the gallbladder in asymptomatic as well as diseased animals. The ability to grow transiently in this location may not only allow possible escaping the immune system but also permit secretion via bile into the intestine to reinfect the same animal or be transmitted. Similar growth in human gallbladders may allow human listeriosis to spread unknowingly in a manner similar to that of typhoid fever.

The *L. monocytogenes* EGDe bile salt hydrolase (*bsh*) gene that is involved in bile tolerance in vitro is positively regulated by PrfA, the principal regulator of *Listeria* virulence genes [162]. Deletion of *bsh* results in reduced faecal carriage after oral infection of guinea pigs, and reduced virulence and liver colonization after intravenous inoculation of mice [162]. We have also shown that mutation of either *L. monocytogenes* LO28 *bsh* or *btlB* (*lmo0754*) can reduce bile tolerance and faecal carriage in mice [163, Begley et al., submitted for publication]. Interestingly, the gene encoding a putative penicillin V amidase (*lmo0447*) contributes to bile tolerance in vitro but not to murine colonization and is absent from certain pathogenic strains of *L. monocytogenes* [163].

7.3. Bile and virulence conclusions

The ability to respond to environmental signals present in the host and to modulate virulence gene expression accordingly is a requirement of a successful enteric pathogen. It is now clear that bile can act as an environmental signal in the same way as classical signals such as pH, osmolarity and temperature. In addition, pathogens can respond to bile in contradictory fashions to facilitate their own pathogenic mechanisms, e.g. bile represses *Salmonella* invasion but increases invasion of *Shigella*.

8. Conclusions and potential benefits of bile research

Bile represents a major challenge to the survival and subsequent colonization of microorganisms in the gastrointestinal tract. It is evident that certain bacteria have evolved to resist its actions and pathogens can even use bile to their advantage to regulate virulence determinants. However, compared to other physiological stresses, bile stress is as yet largely unexplored. Detailed physiological analyses are required to ascertain both the limits and comparative bile tolerance of various bacteria. Future genetic analyses should concentrate on expanding the information available on the molecular mechanisms governing bile tolerance and investigate how bacteria sense bile and regulate the responses it induces. Research should also focus on the effect of bile on virulence factor production and the impact this has on pathogenesis.

Undoubtedly, knowledge gained through bile research will provide further insight into the survival of pathogens in vivo. An understanding of how bacteria can tolerate the high levels of bile encountered in the gallbladder may also explain persistent/chronic infections such as those caused by *S. typhi*. It is possible that information obtained from bile research may be exploited in antibacterial and preventive therapies. Future antimicrobial treatments may involve bile salts and in fact recent studies have demonstrated their effectiveness as topical microbicidal agent for sexually transmitted diseases [257]. Bile-mediated pathogenic mechanisms may also be targeted, however care must be taken not to alter the indigenous gastrointestinal microflora. Compounds could potentially be designed to inhibit the action of bile efflux pumps or target the gallbladder to eliminate carriage of organisms able to cause cholecystitis. The realisation that pathogens use bile to regulate virulence properties highlights the fact that an adequate concentration of bile in the intestine is of utmost importance to human health. Individuals with low levels of bile such as those with biliary or liver abnormalities, deficiencies in enterohepatic circulation, obstructions in intestinal absorption or malnourished individuals can be given bile acids orally (conjugated bile acid replacement therapy) or given hormones to increase bile acid synthesis in the liver.

The connection between bile tolerance and other stresses should be borne in mind by food processors, especially when designing safe, minimally processed products. Exposure to mild stresses may actually "prepare" bacteria to resist the actions of bile when subsequently ingested. Knowledge gained from investigating the interplay between bile stress and other stresses may however aid in the development of better probiotics. Exposing strains to sublethal levels of bile or heterologous stresses before consumption may increase their bile tolerance and thereby improve their survival in the intestine. It may also be possible to manipulate bile-resistance genes associated with colonization or persistence in the intestinal tract. One possible candidate is the bile salt hydrolase gene discussed in Section 6.2. As hydrolysis of bile salts results in increased usage of cholesterol

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to synthesize new bile salts, serum cholesterol levels are lowered. Therefore, in addition to increasing bile tolerance, enhancing the bile salt hydrolase activity of probiotics offers potential as a "biological" alternative to pharmaceutical interventions to treat hypercholesterolaemia.

Therefore, in addition to gaining a better understanding of the disease processes of pathogens, knowledge gained from bile research may ultimately lead to the design of new antimicrobial treatments or assist in the development of improved probiotic strains.

Acknowledgements

The authors would like to acknowledge the funding received from the Irish Government under the National Development Plan 2000–2006 and funding of the Alimentary Pharmabiotic Centre by the Science Foundation of Ireland Centres for Science Engineering and Technology (CSET) scheme.

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