CHAPTER TWO

In Sickness and in Health: The Relationships Between Bacteria and Bile in the Human Gut

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Contents

1. Introduction 44
2. Bile in Digestion 44
   2.1 Makeup of Biliary Secretion 44
   2.2 Synthesis of Bile Salts and Enterohepatic Recycling 45
   2.3 Modification of Bile Salts by the Microbiome 46
3. Bile as a Barrier 47
   3.1 Intestinal Landscape and Niches 48
   3.2 Direct Toxicity Against Microbes 48
   3.3 Indirect Immune Activation 50
4. Bacterial Responses to Bile 50
   4.1 Exclusion of Bile and Bile Salts 51
   4.2 Efflux of Bile 53
   4.3 Repair and Defense 55
   4.4 Virulence-Associated Responses 56
5. Conclusions 57
References 57

Abstract

Colonization of a human host with a commensal microbiota has a complex interaction in which bacterial communities provide numerous health benefits to the host. An equilibrium between host and microbiota is kept in check with the help of biliary secretions by the host. Bile, composed primarily of bile salts, promotes digestion. It also provides a barrier between host and bacteria. After bile salts are synthesized in the liver, they are stored in the gallbladder to be released after food intake. The set of host-secreted bile salts is modified by the resident bacteria. Because bile salts are toxic to bacteria, an equilibrium of modified bile salts is reached that allows commensal bacteria to survive, yet rebuffs invading pathogens. In addition to direct toxic effects on cells, bile salts maintain homeostasis as signaling molecules, tuning the immune system. To cause disease, gram-negative pathogenic bacteria have shared strategies to survive this harsh environment. Through exclusion of bile, efflux of bile, and repair of bile-induced
1. INTRODUCTION

In the gut, trillions of bacteria are kept in balance with the host immune system. Prevention of bacterial outgrowth is coordinated by several immune mechanisms. When a pathogenic bacterium enters this ecosystem, it must also cope with the immune pressure that maintains homeostasis in the gut environment. Bile is one mechanism that functions to maintain this balance that can often be overcome by pathogenic bacteria. Bile is produced by the host as a natural part of digestion but is also a major physical barrier that bacteria must cope with, both pathogenic and commensal. In addition to direct antimicrobial killing, bile components have been recently appreciated to play a role in regulation of immune factors that contribute to bacterial control. This review will consider the role that bile plays in protecting the host during health and disease, as well as the ways in which bacteria respond to bile. Particular focus will be given to the mechanisms by which pathogenic bacteria can survive the antimicrobial activity of bile and, in many cases, use this host compound as a signal to coordinate colonization and virulence.

2. BILE IN DIGESTION

2.1 Makeup of Biliary Secretion

Bile is a complex mixture composed of bile acids, bilirubin, cholesterol, phospholipids, fatty acids (both saturated and unsaturated), and ions such as Ca^{2+}, Na^+, and Cl^− (Hofmann, 1989). The most abundant and most active components in bile are bile acids. Due to the numerous studies of “bile” and component “bile salts” both will be given greater consideration in this review than other bile components. Bile salts are rigid amphipathic molecules with the sterol core. This core is a hydrophobic, with the hydroxyl groups orienting to one side providing a hydrophilic face. In this way, bile salts can act as strong surfactants and have unique interactions with surfaces and membranes relative to other detergents (Hofmann & Small, 1967; Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). One of the most important activities of bile salts is to solubilize lipids. During digestion, this activity helps to solubilize dietary lipids to promote absorption by the intestinal tract.

Bile salts are synthesized in the liver from cholesterol precursors. The two main bile salts synthesized are cholic acid and chenodeoxycholic acid and are
termed primary bile acids (Fig. 1). They share a common sterol core and differentiated by the presence of a hydroxyl group. Secondary bile acids lack the C7 hydroxyl group (Fig. 1). Any of these may be conjugated with glycine or taurine amino groups. Before being released from the liver for storage in the gallbladder, primary bile acids are conjugated with a taurine or glycine group, to form taurocholate or glycocholate, respectively (Swann et al., 2011). A single enzyme performs this conjugation reaction. It is interesting to note that the human enzyme has greater affinity for glycine conjugation, so these bile salts are predominant in the human gut compared to taurine in rodents (Falany, Johnson, Barnes, & Diasio, 1994; Swann et al., 2011).

2.2 Synthesis of Bile Salts and Enterohepatic Recycling

Upon food intake, bile is released from the gallbladder where it is stored into the duodenum. Bicarbonate is also released, resulting in an increase of pH

![Figure 1](image_url) Primary bile salts cholate (cholic acid) and chenodeoxycholate (chenodeoxycholic acid) are synthesized in the liver, differing only by hydroxylation status at the 12C position. Before being stored in the gallbladder for use in digestion, primary bile salts are conjugated to a taurine or glycine group with an amide bond. Cholate conjugated to glycine forms glycocholate (glycocholic acid) and with taurine form taurocholic acid. Deconjugation by gut bacteria can return bile acids to cholate and chenodeoxycholate. Once deconjugated, dehydroxylation by gut bacteria can occur at the 7C position. Dehydroxylation of cholate and chenodeoxycholate result in deoxycholate and lithocholate, respectively.
after deposition of acidic stomach juices. As the bile and dietary matter move down the intestine, mixed micelles with bile salts promote absorption of dietary lipids and cholesterol by intestinal cells (Maldonado-Valderrama et al., 2011). Bile salts are not absorbed here but are reclaimed in the distal intestine. They are then transported to the liver and recycled in a process known as enterohepatic circulation. Any bile salts that have been modified by the gut microbiota (discussed in the following section) will be returned to a glycine- or taurine-conjugated primary bile salt form before being returned to the gallbladder (Dawson, Lan, & Rao, 2009). In this way, bile acids are used several times before being excreted in fecal matter. For an in-depth review of bile physiology see Hofmann (1999).

2.3 Modification of Bile Salts by the Microbiome

Extensive modification of bile acids occurs in both the small and large intestine. The three major ways by which commensal bacteria modify bile salts are through deconjugation, oxidation/reduction, and dehydroxylation. Deconjugation is the removal of the amino side group and is performed by bile salt hydrolase enzymes primarily in the small intestine. Numerous classes of both gram-positive and gram-negative bacteria have been found to have distinct bile salt hydrolases with variable affinity for different bile salts (Ridlon, Kang, & Hylemon, 2006). A bile salt hydrolase has also been found in *Listeria monocytogenes*, an invasive gram-positive pathogen responsible for food-borne disease. Without this enzyme, bacteria have increased sensitivity to bile and decreased colonization in a guinea pig model of infection (Dussurget et al., 2002). Suggested benefits for commensals and pathogens include detoxification of bile acids and metabolism of released amine groups. Hydroxysteroid dehydrogenase enzymes carry out the epimerization of and redox changes to bile salts, resulting in generation of redox equivalents for energy production (Ridlon et al., 2006). Finally, for primary bile salts that evade uptake in the small intestine and reach the large intestine, dehydroxylation at the 7C position will occur, resulting in bacterial-mediated formation of secondary bile salts. This is a multistep process that is thought to occur as the bacteria utilize the bile acids as an electron receptor for respiration (Ridlon et al., 2006). Dehydroxylation occurs in a smaller number of bacteria, typically *Clostridium* sp., but is an efficient process, converting nearly all primary bile salts to secondary (Hirano, Nakama, Tamaki, & Oda, 1981; Narushima et al., 2006; Wells, Berr, Thomas, Dowling, & Hylemon, 2000).

This balance of bile salt abundance and modification is beneficial to both host and microbiome. As discussed, there are multiple growth-related benefits
received by microbial communities as a result of bile acid modification. This homeostasis can also provide stability that prevents invasion by pathogens through colonization resistance (Britton & Young, 2014). As microbes compete for nutrients, commensals well adapted to their niche can block out invaders. Additionally, adaptation to a certain bile acid profile can provide commensals with a benefit that is eliminated when there is a shift in composition. This can occur when there is disruption of the microbiome by antibiotic treatment that alters the abundance of bacteria capable of modifying bile salts (Zhao et al., 2013). Treatment with certain antibiotics is a known risk factor for Clostridium difficile infection, an increasingly important spore-forming nosocomial pathogen. A healthy microbiome is recalcitrant to C. difficile outgrowth, partially because the presence of secondary bile salts prevents growth. Antibiotic treatment depletes other Clostridium species responsible for the formation of secondary bile salts. The hypothesis that this species is protective against C. difficile infection was confirmed when removal of secondary bile acids from the host gut nullified protection (Buffie et al., 2014). Likewise, gnotobiotic animals produce bile salts but have much lower ratios of deconjugated and dehydroxylated bile salts that alter their ability to respond to pathogens (Narushima et al., 2006). Bacterial infection can also modify the composition and homeostasis of bile salt ratios. Infection with S. Typhimurium results in an increase in total bile salt levels in the intestinal lumen. Although the mechanism is not fully understood, it was suggested that increased bile salts provide an advantage to this higher bile tolerance pathogen over commensals (Crawford et al., 2012).

3. BILE AS A BARRIER

The intestinal tract is one of the main interfaces between the human body and the microbes that colonize it. Because it comes in contact with both commensal bacteria and potential pathogens, the intestinal tract is an important immune site. This interface consists the intestinal epithelium covered in mucous layers of decreasing density as they extend into the lumen (Johansson et al., 2011). There is dedicated monitoring by immune cells as well as secretion of antimicrobial compounds such as antimicrobial peptides and antibodies (For further review see Perez-Lopez, Behnsen, Nuccio, & Raffatellu, 2016). Bile is secreted to aid in digestion but also has important immune functions. It regulates bacterial growth both directly through its toxic activity and indirectly through immune signaling.
3.1 Intestinal Landscape and Niches

Bile is released in the duodenum at the proximal small intestine, and bile salts peak in concentration in the proximal ileum (Eastwood & Boyd, 1967). Progressing distally along the small and eventually large intestine, bile salt concentration decreases as bile salts are reabsorbed, altered by bacteria, and incorporated into fecal matter (Hofmann, 2013). Consequently, as bile acid concentration decreases distally, bacterial density increases. Both the concentration as well as relative composition of bile salts present changes distally. Taurine- and glycine-conjugated bile salts are in highest abundance in the proximal small intestine, making up a large fraction of the bile acid milieu (Hofmann, 1999). As bile acids are altered by the microbiome and recirculated, the ratio of primary and conjugated bile acids decreases as secondary, tertiary, and unconjugated bile acids increase. In the large intestine, nearly all bile acids are dehydroxylated by microbial enzymes (Ridlon et al., 2006). Additionally, bile salts are thought to decrease in concentration from the lumen toward the intestinal epithelium due to the layers of mucous secreted by host cells. This decrease in bile salts into the mucosal layer is accompanied by an increase in bicarbonate toward the epithelium (Flemstrom & Isenberg, 2001). These spatial differences in bile and other intestinal compounds create a complex architecture of signals, stressors, and environments for bacteria to navigate.

3.2 Direct Toxicity Against Microbes

Bile has direct antimicrobial activity that can slow the growth of or kill bacteria. Because bile salts solubilize lipids during digestion, it is typically thought that cell death occurs from membrane disruption. While membrane damage does occur, it has become clear that bile salts can act on several processes that may contribute to its inhibitory effect. Early studies with erythrocytes demonstrated that bile acids could indeed disrupt membranes, causing leakage and lysis (Sagawa, Tazuma, & Kajima, 1993; Yasuhara, Tonooka, Kamei, & Sakamoto, 1985). Indirect evidence for membrane damage by bile acids is provided by analysis of membrane composition before and after bile exposure. In gram-positive Bifidobacteria animalis ssp., exposure to bile affects ratios of phospholipids and proteins in the membrane resulting a decrease in membrane fluidity and increase in bile tolerance (Ruiz, Sanchez, Ruas-Madiedo, Reyes-Gavila De Losn, & Margolles, 2007). Finally, alteration of inner membrane can sensitize gram-negative bacteria like Salmonella enterica to bile salts like...
deoxycholate (López-Garrido, Cheng, García-Quintanilla, Portillo, & Casadesús, 2010).

Once bile acids enter cells, they can cause damage to proteins, DNA, and RNA. Bile salts can denature proteins through their detergent activity, causing protein dysfunction and aggregation. For gram-positive *Lactobacilli* and *Bifidobacteria*, when bile salts accumulate in the cell cytoplasm, they cause acidification and therefore a dissipation of the proton gradient, resulting in halted bacterial growth. The concentration of bile salts to reach this threshold aligns with the minimum inhibitory concentration of the bile acids, with secondary bile salt deoxycholate being much lower than cholate (Kurdi, Kawanishi, Mizutani, & Yokota, 2006). When exposed to bile, gram-negative *Vibrio cholerae* activates certain chaperone proteins, the mutants of which are defective for growth in bile (Cremers, Knoefler, Vitvitsky, Banerjee, & Jakob, 2014). Without this chaperone, bile acids caused the aggregation of many proteins, including those for RNA transcription and protein translation, demonstrating how disruption of one pathway can affect many cellular processes.

Exposure to bile also induces redox stress in gram-positive and gram-negative bacteria. Bile induces redox-protective proteins like DinF in *Escherichia coli* and OxyR and SoxS in *S. enterica* (Prieto, Ramos-Morales, & Casadesús, 2006; Rodríguez-Beltrán, Rodríguez-Rojas, Guelfo, Couce, & Blázquez, 2012). This redox stress can upset disulfide bonds, leading to protein misfolding and aggregation. Oxidative stress caused by bile salts is also thought to contribute to DNA damage. For many bacteria, RecA plays a role in DNA repair, including induction of the SOS repair response and contributing to homologous recombination. The fact that RecA and downstream DNA repair mutants are impaired for growth in bile also demonstrates that bile leads to DNA damage. This is true for pathogens such as *Listeria monocytogenes, E. coli* and *S. enterica* (Badie, Heithoff, Sinsheimer, & Mahan, 2007; Nair, Davis, Shami, & Lagerholm, 2000; Prieto et al., 2006; Rodríguez-Beltrán et al., 2012; van der Veen & Abee, 2011). Direct damage of DNA has been shown in *S. enterica*, where bile increased GC to AT transitions (Prieto, Ramos-Morales, & Casadesús, 2004).

It is also important to remember that bile salts do not equally cause harm. Deconjugated bile salts are typically more toxic to cells. To enter the cell cytoplasm of gram-negative bacteria, bile acids must pass through the outer membrane, which may occur through slow diffusion or more likely through porins in the membrane (Nikaido, 2003). For the plasma membrane of gram-positive and gram-negative bacteria, bile acids can insert into the outer
hemileaftet and then “flip-flop” into the inner hemileaftet. More hydrophobic acids like deoxycholate “flip-flop” most quickly, with unconjugated primary acids like cholate doing so slowly. Conjugated bile acids show little traversal to the inner hemileaftet in experiments using unilamellar vesicles (Donovan & Jackson, 1997; Kamp, Hamilton, & Westerhoff, 1993). This observation was thought to be due to pKa values that would expect deconjugated bile acids more likely to be protonated and able to pass into membranes. Similarly, deoxycholate was shown to cause a greater amount of proton leak than cholate. Fatty acids found in bile also exhibited much more rapid flip-flop than any of the bile acids tested (Kamp et al., 1993).

3.3 Indirect Immune Activation

While the direct bactericidal activity of bile has long been studied, it has more recently been appreciated that bile salts also have an immunomodulatory function. It has long been known that disruption of bile flow to the small intestine can result in outgrowth of bacteria in the proximal small intestine (Bauer et al., 2001; Slocum, Sittig, Specian, & Deitch, 1992). Loss of control was thought to be due to lack of bactericidal effects of bile. A paradox was presented, however, because the conjugated bile salts found in the small intestine have relatively low toxicity. It was subsequently demonstrated that these conjugated bile acids act as a signal for the farnesoid X receptor (FRX), which in turn regulates several mucosal immunity genes (Inagaki et al., 2006). Regulation via FRX is important for protection against bacterial outgrown under dysbiosis that results in altered bile content and likely serves a role in maintaining homeostasis between host immune system and microbiota. Since this discovery, bile salts have been found to act in a signaling role for many physiologic processes including regulation of bile salt synthesis, glucose metabolism, and energy expenditure (Copple & Li, 2016).

4. BACTERIAL RESPONSES TO BILE

For enteric pathogens, it is inevitable that an encounter with bile will occur. Studies examining how bacterial diversification in the gut evolves found that competition for nutrients and presence of bile salts were two of the strongest drivers of selection and diversification (de Paepe et al., 2011). It is not surprising, therefore, that bacteria have developed a myriad
of responses to bile and bile salts. For pathogens, these responses can be summarized into four main strategies: exclusion of bile, extrusion of bile, repair and defense against damage, and modulation of virulence.

These four general themes emerge over the body of research investigating bile and enteric pathogens. Most prominent among the literature are studies involving *E. coli*, *V. cholerae*, and the various *Salmonella* strains. Conveniently, these three bacteria cover several modes of infection by gram-negative enteric pathogens. *E. coli* is a commonly found commensal bacteria that can become pathogenic when conditions are conducive or when certain virulence factors are acquired, such as shiga toxin. *E. coli* stains can be enteroinvasive, can adhere to cells, or indirectly damage the host through use of toxins (Johnson & Nolan, 2009). Pathogenic *V. cholerae* is noninvasive and causes disease primarily though expression of the cholera toxin. *V. cholerae* but can also persist in a nonvirulent state adapted to an aquatic environment (De, 1959; Kamruzzaman et al., 2010). *Salmonella enterica* is an invasive bacteria that relies on its type III secretion system encoded on the *Salmonella* pathogenicity island 1 (SPI1) (Mills, Bajaj, & Lee, 1995). *Salmonella* can also persist asymptotically in the gallbladder as biofilms on gallstones (Prouty, Schwesinger, & Gunn, 2002).

### 4.1 Exclusion of Bile and Bile Salts

Gram-negative bacteria are considered to have some inherent resistance to bile. This is in part due to their outer membrane, which acts as a selective permeability layer. Diffusion through the outer membrane is slow due to its asymmetric nature (Nikaido, 2003). While the inner hemileaflet is largely phospholipid, the outer hemileaflet is heavily decorated with lipopolysaccharide (LPS). Lipid A moieties in the outer membrane anchor a core polysaccharide chain that extends into an O-antigen polysaccharide (Raetz, Reynolds, Trent, & Bishop, 2007; Silhavy, Kahne, & Walker, 2010). As extensively reviewed in the study by Nikaido (2003), the outer membrane provides a barrier through which permeability is slowed or prevented. Molecules largely pass through the outer membrane via porins, integral outer membrane protein channels (Nikaido, 2003). Bacteria that are deficient for LPS or Lipid A are acutely susceptible to bile salts as bile can more easily access the cell where it can cause damage (Murata, Tseng, Guina, Miller, & Nikaido, 2007). *E. coli* with aberrant modification of Lipid A due to *pmrA* regulator overexpression are highly sensitive to bile salts (Froelich, Tran, & Wall, 2006). LPS is also important for *Salmonella* resistance to bile. Loss of the O-antigen results in bile sensitivity, while mutants with very long
O-antigen were found to have a higher minimal inhibitory concentration of deoxycholate, conferring a selective advantage in a mouse model of infection (Crawford et al., 2012; Lacroix, Avoyne, Pinault, Popoff, & Pardon, 1995). More recently, alterations in peptidoglycan structure have also been shown to enhance bile resistance, presumably through further decreasing diffusion of bile salts into the cell. Structure was correlated with protection, and proteins responsible for modification were induced by sublethal concentrations of bile (Hernández et al., 2015).

While the outer membrane itself provides some protection against bile by preventing entry, specific composition of the outer membrane can further enhance exclusion of bile from the cells. When exposed to bile, many gram-negative bacteria will alter the porins present in their outer membrane. These beta barrel proteins are aqueous–filled channels that allow passive diffusion of molecules with little substrate specificity (Rigel & Silhavy, 2012). Many described bacteria have a primary porin or set of porins. Often of larger diameter, bile components can typically pass through these primary porins, prompting bacteria to instead express narrower versions to prevent bile from entering the cell. For *V. cholerae*, OmpT is one of the primarily expressed porins. Upon bile exposure, OmpT expression is repressed while OmpU is reciprocally activated by regulator ToxR. Cells forced to activate OmpT in the place of OmpU due to swapped promoters had decreased bile resistance and greatly diminished colonization (Provenzano & Klose, 2000). Further exploration of these two porins showed that cells expressing the bile-induced OmpU had decreased outer membrane permeability and better restricted anionic flux (Simonet, Baslé, Klose, & Delcour, 2003; Wibbenmeyer et al., 2002). Similarly, in *Salmonella* spp. and *E. coli*, the narrower OmpC channel is favored in the presence of bile over the wider OmpF (Nikaido, 2003; Thanassi, Cheng, & Nikaido, 1997). Exclusion of bile can provide cross protection to antibiotics in clinical isolates. Tetracycline-resistant *S. typhi* isolates lacked proper expression of OmpF porins, and other isolates lacked porins altogether (Nikaido, 2003; Toro, Lobos, Calderon, Rodriguez, & Mora, 1990).

Bacteria can also attempt to physically block out bile by forming biofilms. These are bacterial communities encased in a self-produced exopolysaccharide matrix. Often this matrix can decrease access of bile acids to cells. Many bacteria naturally form biofilms as a part of normal growth in response to stress or nutrient limitation and can also be induced by bile in some bacteria. Bile salts can enhance or induce biofilm formation for many pathogens, including *V. cholerae*, *Campylobacter jejuni*, *Salmonella*
spp., and *Klebsiella pneumoniae* (Chen et al., 2014; Hung, Zhu, Sturtevant, & Mekalanos, 2006; Prouty et al., 2002; Svensson, Pryjma, & Gaynor, 2014). Bile can also enhance biofilm formation of commensal gram-positive bacteria such as *Bacteroides fragilis* (Pumbwe et al., 2007).

### 4.2 Efflux of Bile

Once bile acids have passed through the outer membrane or when encountering gram-positive bacteria, bile components such as bile salts and fatty acids reach the inner membrane. Because of their detergent nature, bile acids can insert into or pass through the inner membrane. This occurs much more efficiently than passage through the outer membrane and does not rely on porins or other proteins (Donovan & Jackson, 1997). Fatty acids can also directly integrate into the membrane (Kamp et al., 1993). When blocking entry of bile is not sufficient, cells must actively transport these harmful substances back out into the environment. Gram-negative bacteria have well-documented means of performing active efflux to remove bile salts, but bile removal strategies are less well defined for gram-positive bacteria.

Gram-negative bacteria can efficiently pump bile salts and other toxic compounds out of the cell by use of the resistance–nodulation–division (RND) family of transporters. These three-member pumps traverse the inner membrane, periplasm, and outer membrane, extruding substrates from the cytoplasm into the environment. The first and most well-studied member, AcrAB, was first described in *E. coli* (Ma et al., 1993). AcrA acts as a periplasmic adapter for AcrB, the inner membrane pump with a sizable periplasmic component. These work in complex with TolC outer membrane channel (Nikaido, 2012). Deletion of any one of these components renders *E. coli* susceptible to many harmful compounds, including bile salts (Rosenberg, Bertenthal, Nilles, Bertrand, & Nikaido, 2003).

AcrAB–TolC in *E. coli* can export a wide variety of substrates and as such is considered a multidrug efflux pump (Zgurskaya & Nikaido, 1999). This family is generally known for its wide substrate specificity, but certain members do have higher affinity for specific compounds. For example, *V. cholerae* VexCD (also named BreAB) was shown to have increased sensitivity to deoxycholate but not to other tested compounds (Bina, Provenzano, Wang, Bina, & Mekalanos, 2006). Indeed, studies attempting to understand substrate specificity for RND pumps have shown that conjugated bile salts such as taurocholate have the strongest affinity and may be the natural substrate for *E. coli*. This makes sense considering that *E. coli* lives in an environment rich in bile salts (Thanassi et al., 1997; Zgurskaya & Nikaido, 1999).
Efflux pumps such as AcrAB are constitutively expressed in many bacteria, but bile and other insults can either increase expression or promote expression of axillary pumps that may have higher specificity for bile (Chatterjee, Chaudhuri, Saha, Gupta, & Chowdhury, 2004). *V. cholerae* VexAB and VexCD were both induced by 0.2% bile, and VexCD was shown specifically to be induced by regulator BreR in response to deoxycholate (Bina et al., 2006; Cerda-Maira, Kovacikova, Jude, Skorupski, & Taylor, 2013; Cerda-Maira, Ringelberg, & Taylor, 2008). Both efflux pumps were also induced in rabbit ileal loop model, indicating that these genes are likely contributing to survival during infection (Xu, Dziejman, & Mekalanos, 2003). AcrAB homologs are typically encoded as an operon, with TolC being encoded elsewhere on the chromosome, and often paired with multiple pumps (Baucheron et al., 2014; Fralick, 1996). In *V. cholerae*, multiple RND family pumps are thought to pair with the same TolC, as deletion of this outer membrane protein renders the bacteria completely sensitive to bile salts, while other putative TolC homologs do not (Bina & Mekalanos, 2001). While AcrAB type pumps are the workhorse for bile extrusion, auxiliary pumps in other families contribute to bile efflux. In *E. coli*, MdtM is a single component pump of the major facilitator superfamily whose affinity for bile salts resulted in a synergistic bile resistance with AcrAB pumps (Paul et al., 2014).

Other pathogens such as *E. coli* and *Salmonella* spp. also rely on efflux pumps as main source of protection against bile (Fralick & Burns-Keliher, 1994; Prouty et al., 2002). *C. jejuni* is a gram-negative bacteria that colonizes the distal ilium and colon and is a major source of food poisoning. The *C. jejuni* homolog of AcrAB-TolC, CmeABC, confers resistance to many bile salts as well as intestinal extracts from chickens (Akiba, Lin, Barton, & Zhang, 2006; Pumbwe, Randall, Woodward, & Piddock, 2004). Likewise, CmeB and CmeC mutants were unable to colonize the chicken intestine. Expression of this pump is constitutive, and during colonization, chickens develop antibodies to CmeABC, suggesting its importance in vivo (Lin, Sahin, Michel, & Zhang, 2003). These gram-negative pathogens share a similar regulatory circuit to induce RND family pumps. A repressor (CmeR, BreR, RamR for *C. jejuni*, *V. cholerae*, or *S. Typhimurium*) binds to a promoter to prevent transcription of a pump or pump activator. In the presence of bile or specific bile acids, repression is released, allowing for direct induction of efflux pumps (CmeABC and BreAB) or indirect induction via a transcriptional activator (RamA induces AcrAB) (Baucheron et al., 2014; Cerda-Maira et al., 2013, 2008; Pumbwe, Randall, Woodward, & Piddock, 2005).
As discussed previously, the outer membrane of gram-negative bacteria provides some barrier to entry of bile components. However, bile sensitivity of RND family pump-deficient bacteria demonstrates that the barrier is insufficient to provide full protection. RND pumps are somewhat unique, in that substrates are not simply moved from the cytosol to the periplasm but are removed from the cell where they will again diffuse more slowly through the outer membrane. This synergistic approach helps gram-negative bacteria survive higher bile concentrations (Koronakis, Eswaran, & Hughes, 2004).

### 4.3 Repair and Defense

To respond to the bile insult, bacteria will attempt to exclude and export bile. However, bile components do ultimately enter the cell. To successfully grow in this environment, bacteria must respond to the damage caused by bile. By understanding which genes are induced in response to bile, it has been possible to infer the types of damage that have been inflicted. As discussed previously, this can include membrane damage, protein denaturing or aggregation, and DNA damage (Begley, Gahan, & Hill, 2005). These bacterial processes may occur through ROS-based damage and other direct detergent activity.

Therefore, many bacteria induce genes that are responsible for ROS and DNA damage repair. For *Salmonella* spp., mutants defective for several methods of DNA repair are sensitive to bile. This includes those for base excision repair, SOS response, and homologous recombination (Prieto et al., 2004, 2006). In response to bile, *Salmonella* induces not only DNA damage repair but many general stress-response genes under the control of RpoS (Hernández, Cota, Ducret, Aussel, & Casadesús, 2012). LexA-regulated error prone-repair gene, *impB*, was both induced by and required for survival in bile in *Enteroaggregative E. coli* (Joo, Macfarlane-Smith, & Okeke, 2007). In *E. coli*, DinF was both protective against bile via reduction of oxidative stress and was found to be regulated by LexA (Rodríguez-Beltrán et al., 2012). In *L. monocytogenes*, DNA repair was both induced by bile and required for bile resistance and invasion (van der Veen & Abee, 2011).

These defensive responses are often induced at the same time as those that attempt to block or remove bile. At subinhibitory concentrations of bile, bacteria may still induce defensive responses. In this way, they can be “primed” to withstand higher concentrations of bile. For example, the outer membrane of *V. cholerae* is more permeable than that of *E. coli* as indicated by novobiocin sensitivity. Following incubation with low levels of bile, *V. cholerae* membrane permeability decreased 10-fold, while *E. coli* permeability had no further
change (Chatterjee et al., 2004). Priming of S. enterica with moderate amounts of bile can increase bile tolerance by 14% (Hernández et al., 2012).

4.4 Virulence-Associated Responses

In addition to survival strategies, many pathogenic bacteria have adapted to translate their encounter with bile acids into modulation of a virulence program. In response to bile, bacteria can regulate virulence activity positively, negatively, or a combination of the two. In C. jejuni, many virulence genes are positively regulated by bile (Doig, Yao, Burr, Guerry, & Trust, 1996; Malik-Kale, Parker, & Konkel, 2008). Deoxycholate specifically promoted expression of major virulence factors including the Campylobacter invasion antigens, leading to more rapid invasion of epithelial cells. Bile consistently represses virulence-associated genes in Salmonella. In particular, bile decreased invasion of epithelial cells in vivo (Prouty & Gunn, 2000). This can be at least partially attributed to the repression of virulence-related genes and requisite motility genes, as both SPI1 and SPI2 pathogenicity islands were downregulated by bile (Hernández et al., 2012; Prouty et al., 2004). This repression has been suggested to result in delay of virulence expression in the bile salt–rich lumen until bacteria can access the epithelium (Prouty & Gunn, 2000).

V. cholerae has complex responses to bile and serves as a reminder of the heterogeneous nature of bile. Original studies suggested that bile inhibits virulence. In the O1 classical-biotype strain O395, which has high production of cholera toxin (CT), crude bile decreased toxin production (Gupta & Chowdhury, 1997). Subsequently, in this same strain, it was found that no individual bile salt decreased CT production, leading to the discovery that fatty acids repress virulence through binding directly with regulator ToxT (Chatterjee, Dutta, & Chowdhury, 2007; Hung & Mekalanos, 2005; Plecha & Withey, 2015). This same study suggested that CT could actually be induced directly by ToxR in the presence of cholate for O1 classical-biotype (Hung & Mekalanos, 2005). In the El Tor biotype, secondary and unconjugated bile acids such as cholate and deoxycholate do not promote virulence. However, the conjugated primary bile salts taurocholate and glycocholate do promote virulence in El Tor biotype (Yang et al., 2013). In fact, the former two bile salts stimulate growth as a biofilm, which is antithetical to virulence within the host (Hung et al., 2006). Bile has also been shown to promote motility, which is required for efficient colonization (Butler & Camilli, 2009; Gupta & Chowdhury, 1997). Considering the locations along the intestine that these different bile salts may be found,
we can appreciate that each of these different compounds and combination of compounds likely represent a spatiotemporal point within infection for the bacteria. For example, the abundance of conjugated bile acids in the proximal small intestine may promote motility and virulence to drive colonization and virulence. More distally, an abundance of secondary bile acids may signal impending exit from the host and a transition to expression of genes to survive outside the host.

5. CONCLUSIONS

While bile is a complex mixture, it is interesting to consider why exposure to different individual bile salts can have drastically different phenotypic outcomes within the same bacteria. One possible reason is that the bile salts can signal directly or indirectly through cellular stress. Bile salts can act as an environmental signal for many pathogens. For example, conjugated bile salts such as taurocholate and glycocholate can initiate growth of spores for *C. difficile* (Francis, Allen, Shrestha, & Sorg, 2013). For *V. cholerae*, these bile salts promote virulence induction and taurocholate enhances biofilm egress (Hay & Zhu, 2015; Yang et al., 2013). However, the secondary bile acid deoxycholate has an opposite effect on these phenotypes. It damages *C. difficile* to the point of preventing infection (Buffie et al., 2014). For *V. cholerae* deoxycholate does not induce virulence and promotes entry into a biofilm state.

The reasons for these differences may involve adaptation to different signals as a proxy for different niches. Differential phenotypes may also be a product of chemical and physical characteristics of the bile acids themselves. For example, conjugated bile salts are less toxic to cells and in higher relative abundance in the small intestine. Therefore, they may serve as a better signal molecule to alert bacteria to entry into the intestinal environment. Deoxycholate is more damaging to cells and secondary bile salts are relatively more abundant in the large intestine, suggesting that these bile salts may either act as a different spatial cue or may more rapidly cause damage that is detected by cells. It is not surprising that within this landscape, pathogens can fine tune their responses to these conditions to find the optimal niche for survival and virulence induction.

REFERENCES

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