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Bile-induced peptidoglycan remodelling in *Salmonella enterica*

Sara B. Hernández,[1] Felipe Cava,[2†]
M. Graciela Pucciarelli,[2]
Francisco García-del Portillo,[3] Miguel A. de Pedro[2]
and Josep Casadesús[1*]
[1]*Facultad de Biología, Departamento de Genética, Universidad de Sevilla, Apartado 1095, 41080 Sevilla, Spain.*
[2]*Centro de Biología Molecular Severo Ochoa, CSIC-Universidad Autónoma de Madrid, Madrid, Spain.*
[3]*Centro Nacional de Biotecnología (CNB)-CSIC, Madrid, Spain.*

Summary

Changes in the peptidoglycan (PG) structure of *Salmonella enterica* are detected in the presence of a sublethal concentration of sodium deoxycholate (DOC): (i) lower proportions of Braun lipoprotein (Lpp)-bound muropeptides; (ii) reduced levels of muropeptides cross-linked by L(meso)-diaminopimelyl-D(meso)-diaminopimelic acid (L-D) peptide bridges (3-3 cross-links). Similar structural changes are found in *S. enterica* cultures adapted to grow in the presence of a lethal concentration of DOC, suggesting that reduced anchoring of Braun protein to PG and low occurrence of 3-3 cross-links may increase *S. enterica* resistance to bile. This view is further supported by additional observations: (i) A triple mutant lacking L,D-transpeptidases YbiS, ErfK, and YcfS, which does not contain Lpp anchored to PG, is hyper-resistant to bile; (ii) enhanced 3-3 cross-linking upon overexpression of YnhG transpeptidase causes a decrease in bile resistance. These observations suggest that remodelling of the cell wall may be added to the list of adaptive responses that permit survival of *S. enterica* in the presence of bile.

Received 14 February, 2014; revised 11 April, 2014; accepted 20 April, 2014. *For correspondence. E-mail casadesus@us.es; Tel. +34 95 542 0881. Fax +34 95 455 7104. †Current address: Department of Molecular Biology and Laboratory for Molecular Infection Medicine Sweden, Umeå Centre for Microbial Research, Umeå University, Umeå 90187, Sweden.

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Introduction

Bile is a fluid containing cholesterol, bile salts, phospholipids, proteins, bilirubin and a variety of electrolytes (Hofmann, 1998). Bile is synthesised by parenchymal cells (hepatocytes) in the liver (Hofmann, 1998). A fraction of bile flows directly into the small intestine, while another fraction is diverted to the gall bladder. During food passage, the enteric hormone cholecystokinin induces gall bladder contraction (Hofmann, 2001). Release of bile aids in the digestion of fats, facilitates absorption of fat-soluble vitamins in the intestine, and contributes to the elimination of excess cholesterol and waste metabolic products produced in the liver (Hofmann, 2001; Hofmann and Hagey, 2008).

About two-thirds of bile (dry weight) are made of bile salts, a family of molecules with steroid structure that are derived from cholesterol (Hofmann, 1999). Aside from their role in digestion, bile salts have additional physiological activities as transcriptional regulators of mammalian genes involved in cholesterol metabolism (Redinger, 2003; Lefebvre *et al.*, 2009). Bile salts also regulate the expression of specific bacterial genes and may be considered signals used by enteric bacteria to identify the intestinal environment (Gunn, 2000). However, bile salts are also antibacterial compounds that disrupt membranes, denature proteins and cause oxidative damage to DNA (Gunn, 2000; Prieto *et al.*, 2004; 2006; Begley *et al.*, 2005; Merritt and Donaldson, 2009). At high concentrations, bile salts are bactericidal (Hernandez *et al.*, 2012).

Bacterial species adapted to the mammalian intestine are resistant to the antibacterial activity of bile salts, a trait exploited for the design of selective microbiological media such as the one-century-old MacConkey agar used in the identification of genera of the family *Enterobacteriaceae*. A relevant example of bile-resistant bacterial species is *Salmonella enterica*: During systemic infection, *Salmonella* colonises the hepatobiliary tract including the bile-laden gall bladder (Baumler *et al.*, 2011; Gonzalez-Escobedo *et al.*, 2011). In chronic carriers of typhoid, the gall bladder is the major niche for *Salmonella* Typhi (Gonzalez-Escobedo *et al.*, 2011), sometimes forming biofilms on gallstones (Crawford *et al.*, 2010).

Bile resistance can be studied under laboratory conditions by supplementing microbiological culture media with either ox bile or individual bile salts (Casadesus *et al.*, 2010). Using this reductionist approach, genetic and biochemical analyses have permitted the identification of cell components and mechanisms involved in bacterial resistance to bile (Gunn, 2000; Casadesus *et al.*, 2010). For instance, envelope structures play a major role in bile resistance by providing barriers that reduce bile salt uptake. Such structures include the lipopolysaccharide (LPS) (Picken and Beacham, 1977; van Velkinburgh and Gunn, 1999; Crawford *et al.*, 2012; May and Groisman, 2013) and the enterobacterial common antigen (Ramos-Morales *et al.*, 2003). Choice of bile-insensitive porins (Thanassi *et al.*, 1997; Wibbenmeyer *et al.*, 2002) and downregulation of genes encoding bile-sensitive porins (Hernandez *et al.*, 2012) may also reduce uptake of bile salts. Furthermore, efflux systems decrease the intracellular concentration of bile salts (Thanassi *et al.*, 1997; Piddock, 2006). While the involvement of these envelope structures in bile resistance has been confirmed by numerous studies (Ruiz *et al.*, 2007; Merritt and Donaldson, 2009; Casadesus *et al.*, 2010; Payne *et al.*, 2013), the potential involvement of the PG had not been investigated previously.

The envelope structure is similar in *S. enterica* and *E. coli* (Glauner *et al.*, 1988; Gally and Cooper, 1993). A major component is the peptidoglycan sacculus (PG, also called murein), which is located in the periplasmic space and provides both mechanical strength and cell shape (Vollmer *et al.*, 2008; Page, 2012). PG is a heteropolymer, consisting of glycan strands of N-acetylmuramic acid and N-acetylglucosamine connected through short peptide cross-links (Dramsi *et al.*, 2008). Two different types of peptide cross-links can be distinguished: the most common 4-3 cross-links formed by D,D-transpeptidases and 3-3 cross-links formed by L,D-transpeptidases (Glauner *et al.*, 1988; Magnet *et al.*, 2007). The PG is linked covalently with the outer membrane by Braun lipoprotein (Lpp), which forms trimers, of which only one subunit is anchored to PG (Hirashima *et al.*, 1973; Braun, 1975). In *E. coli*, lack of Lpp alters the permeability barrier of the outer membrane increasing bacterial susceptibility to detergents (Hirota *et al.*, 1977). In *Klebsiella pneumoniae*, lack of Lpp causes virulence-related defects including sensitivity to bile salts (Hsieh *et al.*, 2013). In *Salmonella enterica* serovar Typhimurium, Lpp is redundantly encoded by two chromosomal genes (*lppA* and *lppB)*, and mutants lacking both Lpp species are severely attenuated upon mouse infection (Sha *et al.*, 2004).

Previous studies have shown that the chemical structure of the PG can change depending on the growth phase of the culture (Pisabarro *et al.*, 1985; Cava *et al.*, 2011), during infection of eukaryotic cells (Quintela *et al.*, 1997), and also in response to environmental conditions such as the presence of D-amino acids (Caparros *et al.*, 1992; Kenyon *et al.*, 2007). Furthermore, a recent study had shown that exposure of *S. enterica* to bile induces changes in the amount and/or the activity of penicillin-binding proteins (Hernandez *et al.*, 2013), thereby raising the possibility that PG remodelling might occur in the presence of bile. This study shows that exposure to the archetypal bile salt sodium deoxycholate (DOC) is associated with changes in the PG structure of *S. enterica* serovar Typhimurium, and that PG remodelling contributes to bile resistance.

Results

Growth of S. enterica *in the presence of a sublethal concentration of sodium deoxycholate alters peptidoglycan structure*

The effect of sodium deoxycholate on PG structure was analysed in *S. enterica* cultures grown in Luria–Bertani (LB) containing 5% DOC, a sublethal concentration that permits *S. enterica* adaptation to lethal concentrations (Hernandez *et al.*, 2012). Adaptation involves remodelling of the outer membrane, activation of efflux systems and stress responses, and additional physiological adjustments that remain poorly understood (Hernandez *et al.*, 2012). Muropeptide composition was determined by high-performance liquid chromatography (HPLC). Quantification of each muropeptide species was performed by integration of the peaks of the HPLC profile, and the muropeptides were grouped into classes according to structural similarities (Glauner, 1988). Because *S. enterica* shows different levels of bile sensitivity during exponential growth and in the stationary phase (Pucciarelli *et al.*, 2002), PG analysis was performed under both growth conditions. The main observations were as follows:

(i) A reduction was detected in Lpp-associated monomer (M3L) and dimer (D43L) (Braun and Rehn, 1969). Relative to control cultures, *S. enterica* grown in the presence of 5% DOC underwent a reduction in Lpp-containing muropeptides of approx. 30% in exponential phase (Table 1 and Supporting information Fig. S1) and around 40% in stationary phase (Table 1 and Supporting information Fig. S2). A reduced amount of PG-bound Braun lipoprotein (Lpp*) was detected by Western analysis in the presence of DOC (Fig. 1). In contrast, a similar amount of unbound Braun lipoprotein (Lpp) was detected in the presence and in the absence of DOC (Fig. 1).

(ii) Reduction of DAP-DAP (DAP = diaminopimelic acid) 3-3 cross-links (Glauner *et al.*, 1988) was detected. Cross-link reduction was of 50% in exponential phase cultures (Table 1 and Supporting information Fig. S1) and

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Table 1. Muropeptide composition (relative abundance, mol%) of PG from *S. typhimurium* SL1344 grown in LB and LB containing 5% DOC[a].

	Exponential		Stationary	
Muropeptide group	LB	DOC	LB	DOC
Monomers	67.8 ± 3.4	68.4 ± 3.2	59.7 ± 3.1	68.0 ± 2.8
Dimers	30.3 ± 3.2	29.5 ± 3.0	37.8 ± 3.0	30.2 ± 2.7
Trimers	1.9 ± 0.2	2.1 ± 0.2	2.5 ± 0.3	1.8 ± 0.1
Lipoprotein	9.2 ± 0.9	6.5 ± 0.7	17.0 ± 1.8	10.1 ± 0.8
Anhydro	1.3 ± 0.8	1.5 ± 0.8	2.5 ± 0.9	1.9 ± 1.1
Dap-dap	3.6 ± 1.1	1.6 ± 1.0	9.1 ± 1.5	3.1 ± 0.3
Pentapeptide	0.2 ± 0.2	0.0	0.3 ± 0.4	0.6 ± 0.1
Penta-glycine	0.3 ± 0.2	0.3 ± 0.3	0.0	0.0

a. Averages and standard deviations from three independent experiments.

of 30% in stationary cultures (Table 1 and Supporting information Fig. S2).

A tentative interpretation of these observations was that growth of *S. enterica* in the presence of DOC leads to a decrease both in the amount of Braun lipoprotein anchored to PG and in 3-3 (Dap-Dap) cross-linking.

Adaptation of S. enterica *to survive a lethal concentration of bile is accompanied by changes in Lpp anchoring and cross-linking*

Growth in the presence of sublethal concentrations of bile (e.g. 5% DOC) adapts *S. enterica* to survive a lethal concentration (e.g. 11% DOC) (Hernandez *et al.*, 2012). We thus analysed the muropeptide composition of *S. enterica* grown in LB containing 11% DOC. In these experiments, stationary cultures were used because bile resistance is known to be higher under such conditions (Pucciarelli *et al.*, 2002). Results from a representative experiment are shown in Table 2 and Supporting information Fig. S3. A 50% reduction in the amount of Lpp was detected. DAP-DAP cross-linking was also found to decrease 50%. These observations further support the existence of a correlation between bile resistance and PG remodelling.

Comparison of data from Tables 1 and 2 and Figs S1–S3 also suggest that an inverse correlation may exist between the abundance of Lpp-containing muropeptides and the level of bile resistance: 30% reduction in 5% DOC (exponential culture), 40% reduction in 5% DOC (stationary culture, which shows higher resistance than an exponential culture), and 50% reduction in 11% DOC.

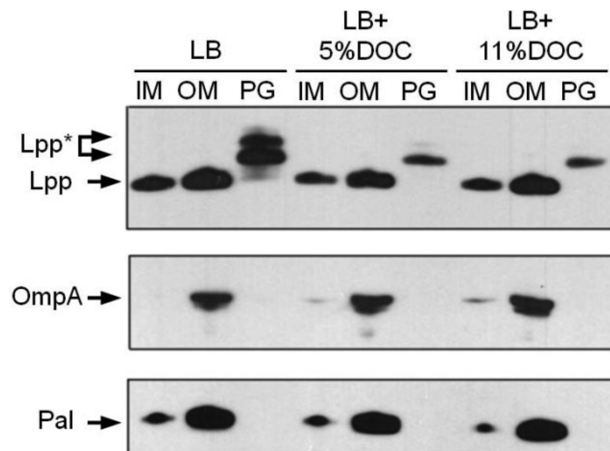

Fig. 1. Western blot analysis of Braun lipoprotein in subcellular fractions of *S. enterica* serovar Typhimurium (IM, inner membrane; OM, outer membrane; PG, peptidoglycan). Cultures were grown in the absence of DOC (LB), in LB containing a sublethal concentration of DOC (5%) and in LB containing a lethal concentration of DOC (11%). Survival under the latter conditions was made possible by previous growth in LB + 5% DOC. Lpp* is Braun lipoprotein anchored to PG, while Lpp is free Braun lipoprotein. OmpA (outer membrane protein A) and Pal (PG-associated lipoprotein) were used as controls.

Increased bile resistance in S. enterica *mutants lacking ErfK, YcfS and YbiS L,D-transpeptidases*

In *E. coli*, anchoring of Lpp to the PG and formation of 3-3 cross-linked muropeptides are catalysed by L,D-transpeptidases (Ldts) (Magnet *et al.*, 2007). Five Ldts exist in *E. coli*: ErfK, YcfS and YbiS, which anchor Lpp to the PG, and YcbB and YnhG, which catalyse 3-3 cross-linking (Magnet *et al.*, 2007; 2008). To further investigate

Table 2. Muropeptide composition of PG from bile-adapted *S. enterica* grown in LB and LB containing 11% DOC.

	Relative abundance (mol%)[a]	
Muropeptide group	LB	DOC
Monomers	63.4 ± 3.1	69.1 ± 2.8
Dimers	34.5 ± 3.0	28.5 ± 3.1
Trimers	2.1 ± 0.3	2.3 ± 0.4
Lipoprotein	14.8 ± 1.8	7.1 ± 1.2
Anhydro	3.5 ± 0.9	3.5 ± 0.8
Dap-dap	6.8 ± 1.5	3.1 ± 1.2
Pentapeptide	0.4 ± 0.4	0.0
Penta-glycine	0.0	0.0

a. Averages and standard deviations from three independent experiments.

Table 3. Minimal inhibitory concentrations of DOC[a].

Strain	Genotype	MIC of DOC (%)
SL1344	wt	7
SV7557	Δ*ybiS*	9
SV7558	Δ*ycfS*	7
SV7559	Δ*erfK*	7
SV7560	Δ*ybiS* Δ*ycfS*	10
SV7561	Δ*ybiS* Δ*erfK*	10
SV7562	Δ*ycfS* Δ*erfK*	7
SV7563	Δ*ybiS* Δ*ycfS* Δ*erfK*	> 14
SV7564	Δ*ycbB*	7
SV7566	Δ*ynhG*	7
SV7567	Δ*ycbB* Δ*ynhG*	7
SV7756	Δ*ybiS* Δ*ycfS* Δ*erfK* p_{BAD}::*ybiS*	7

a. Averages of three or more independent experiments.

the tentative relationship found between muropeptide alteration and bile resistance, *S. enterica* homologues of the *E. coli* Ldt genes (*erfK, ycbB, ycfS, ybiS* and *ynhG*) were deleted in strain SL1344. The minimal inhibitory concentration (MIC) of DOC was then determined in mutants carrying individual or multiple gene deletions. Results shown in Table 3 can be summarised as follows:

(i) Deletion of individual genes *erfK* or *ycfS* did not alter the MIC of DOC (7%). However, a Δ*ybiS* mutant (SV7557) showed increased resistance to DOC (9%).
(ii) The level of DOC resistance of a double mutant Δ*erfK* Δ*ycfS* was similar or identical to that of the wild type (7%). Increased DOC resistance, however, was detected in Δ*ybiS* Δ*ycfS* and Δ*ybiS* Δ*erfK* strains (10%).
(iii) A Δ*ybiS* Δ*ycfS* Δ*erfK* strain (SV7563) showed increased resistance to DOC (> 14% compared with 7% in the wild type).

These observations further support the existence of an inverse relationship between Lpp-PG anchorage and bile resistance. The increased resistance to DOC observed in strains carrying the Δ*ybiS* deletion, alone or combined with Δ*ycfS* and Δ*erfK*, is consistent with the fact that deletion of the *E. coli ybiS* gene severely reduces covalent linkage of Lpp to PG (Magnet *et al*., 2007). However, the most compelling observations were made in the triple mutant Δ*ybiS* Δ*ycfS* Δ*erfK* (SV7563), which displayed hyper-resistance to DOC (Table 3). Viability assays upon exposure to lethal concentrations of DOC (9% and 11%) confirmed that the triple mutant Δ*ybiS* Δ*ycfS* Δ*erfK* was bile-resistant (Fig. 2). Furthermore, partial complementation of the Δ*ybiS* Δ*ycfS* Δ*erfK* mutant with a plasmid-borne *ybiS* gene increased covalent linkage of Braun lipoprotein to PG (Supporting information Fig. S4) and decreased resistance to DOC (Table 3). Because the triple mutant Δ*ybiS* Δ*ycfS* Δ*erfK* does not contain detectable amounts of Lpp-containing muropeptides (Table 4 and Supporting information Fig. S4), these observations support the view that reduction in the amount of Lpp anchored to the PG increases bile resistance in *S. enterica*.

Contribution of 3-3 cross-link reduction to bile resistance

Genetic analysis was also performed to investigate whether reduction of 3-3 cross-linking increased resistance to DOC in *S. enterica*. Neither the single mutants Δ*ycbB* and Δ*ynhG* nor the double mutant Δ*ycbB* Δ*ynhG* showed significant changes in DOC resistance (Table 3). However, these observations did not rule out the possibility that high levels of 3-3 cross-linking could cause bile sensitivity. To address this possibility, the *S. enterica ynhG* gene was cloned on the pBAD18 vector under the control of the arabinose-inducible promoter p_{BAD}. The resulting plasmid (pIZ2019) was introduced in the wild-type, in a mutant devoid of Braun lipoprotein anchored to the PG (Δ*ybiS* Δ*ycfS* Δ*erfK*), and in a mutant lacking cross-link L,D-transpeptidases (Δ*ycbB* Δ*ynhG*).

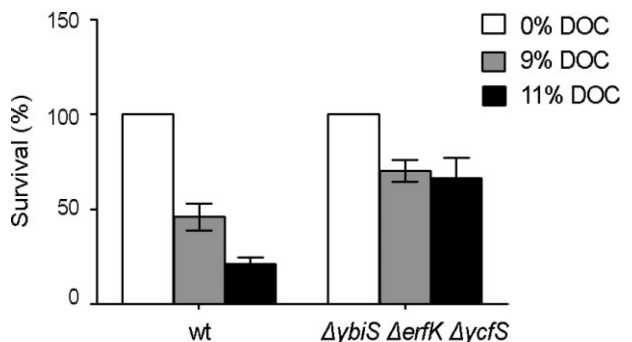

Fig. 2. Viability of SL1344 (wild type) and SV7563 (Δ*ybiS* Δ*ycfS* Δ*erfK*) after exposure to lethal concentrations of DOC (9%DOC and 11% DOC). Both strains showed similar or identical viability in LB. For simplicity, colony numbers are relativised to 100. Histograms represent averages and standard deviations of three independent experiments. Viability differences between SL1344 and SV7563 were significant ($P < 0.05$) both at 9% DOC and at 11% DOC according to Student's *t*-test.

Table 4. Muropeptide composition of PG in *S. typhimurium* SL1344 and in a Δ*ybiS* Δ*ycfS* Δ*erfK* triple mutant (SV7563).

Muropeptide group	Relative abundance (mol%)[a] wt	Relative abundance (mol%)[a] Δ*ybiS* Δ*ycfS* Δ*erfK*
Monomers	64.3 ± 3.1	63.7 ± 0.6
Dimers	33.9 ± 3.0	34.0 ± 0.6
Trimers	1.8 ± 0.3	2.3 ± 0.1
Lipoprotein	13.8 ± 1.8	0.0
Anhydro	3.8 ± 0.9	3.1 ± 0.0
Dap-dap	6.8 ± 1.5	8.0 ± 0.1
Pentapeptide	0.0	0.5 ± 0.2
Penta-glycine	0.0	0.0

a. Averages and standard deviations from three independent experiments.

Table 5. Relative abundance of muropeptides cross-linked through a DAP-DAP peptide bridge (3-3 cross-link) of strains overexpressing (+) or not (–) the *ynhG* L,D-transpeptidase gene and MIC of DOC of these strains.

Strain	*ynhG* overexpression	Relative abundance of 3-3 cross-links (mol%)[a]	MIC of DOC
Wild type	–	4.0 ± 1.0	7
	+	10.7 ± 1.1	4
Δ*ybiS* Δ*ycfS*	–	5.2 ± 0.8	> 12
Δ*erfK*	+	8.3 ± 0.5	7
Δ*ycbB* Δ*ynhG*	–	0.0	7
	+	11.7 ± 1.4	4

a. Averages and standard deviations from 3 independent experiments.

The relative abundance of PG 3-3 cross-links was determined in the presence of pIZ2019 (p_{BAD}::*ynhG*), and the empty vector pBAD18 was included as control. All cultures for muropeptide analysis were grown in LB containing arabinose, and the results can be summarised as follows:

(i) p_{BAD}-driven expression of *ynhG* increased the amount of 3-3 cross-linked muropeptides in all strains (Table 5 and Figs. S5–S7), therefore confirming that the cloned *ynhG* was functional.

(ii) p_{BAD}-driven expression of *ynhG* decreased the MIC of DOC from 7% to 4% in both the wild-type and in the Δ*ycbB* Δ*ynhG* mutant (SV7567), and from > 14% to 7% in the mutant that shows increased resistance to bile and lacks Braun lipoprotein covalently anchored to PG (Table 5).

A tentative interpretation of these observations is that either low levels of cross-linking or absence of cross-linking may be compatible with bile resistance while high levels of cross-linking may be associated with bile sensitivity.

Discussion

The bacterial envelope plays major roles in bile resistance. Mutants of *S. enterica* lacking O antigen chains in the lipopolysaccharide (LPS) are bile-sensitive (Picken and Beacham, 1977), probably because the LPS forms a permeability barrier in the outer membrane (Nikaido, 2003). Bile sensitivity is likewise observed in *S. enterica* mutants lacking lipid A (Froelich *et al.*, 2006) and enterobacterial common antigen (Ramos-Morales *et al.*, 2003). Furthermore, mutations that destabilise the outer membrane (e.g. in the *tolQRA* gene cluster) cause bile sensitivity in both *E. coli* and *S. enterica* (Prouty *et al.*, 2002).

The view of the cell envelope as a passive barrier that reduces bile salt uptake may be, however, simplistic. Several independent studies have reported modifications of the bacterial envelope that contribute to bile resistance. For instance, PhoPQ-mediated remodelling of the lipid A domain of the LPS increases the efficiency of the permeability barrier against antibacterial compounds including bile salts (Murata *et al.*, 2007). The length distribution of O antigen also affects susceptibility to bile salts, raising the possibility that a regulated decrease in the proportion of long O antigen may contribute to bile resistance (May and Groisman, 2013). Because porins provide passage for bile salts (Thanassi *et al.*, 1997), downregulation of *ompC* and *ompF* expression in the presence of bile may also reduce bile salt uptake (Hernandez *et al.*, 2012).

This study adds PG remodelling to the list of adaptive mechanisms that contribute to survival in the presence of bile upon modification of the bacterial envelope. Growth of *S. enterica* in the presence of a sublethal concentration of DOC is accompanied by a reduction in the amount of Lpp anchored to PG (Table 1). Evidence that this reduction is associated with bile resistance is provided by two observations. First, reduced amounts of Lpp-containing muropeptides are found in PG when *S. enterica* is adapted to grow in the presence of a lethal concentration of DOC (Table 2). Second, lack of Lpp anchored to PG causes hyper-resistance to bile (Tables 3 and 4). Because Lpp-containing muropeptides provide covalent linkage between the outer membrane and the PG layer, reduction or loss of this union may increase flexibility in the cell envelope, perhaps altering outer membrane fluidity. In fact, increase of outer membrane fluidity has been shown to permit resistance to stress conditions and antibacterial compounds (Casadei *et al.*, 2002; Jones *et al.*, 2008). Changes in the amount of free Lpp are not detected in the presence of DOC (Fig. 1). Hence, only the amount of PG-bound Lpp seems to be relevant for bile resistance. This observation may not be surprising considering that the free and PG-bound forms of Lpp are found in distinct subcellular locations (Cowles *et al.*, 2011).

Growth of *S. enterica* in the presence of DOC is also associated with a decrease in 3-3 cross-links in the PG, suggesting that this kind of cross-linking may be reduced in the presence of bile (Tables 1 and 2). Somehow surprisingly, a Δ*ycbB* Δ*ynhG* mutant, which lacks 3-3 cross-links, showed a level of DOC sensitivity identical to that of the wild-type (Table 5), suggesting that cross-linking is not actually necessary for bile resistance. However, overproduction of YnhG transpeptidase causes a strong decrease in bile resistance (Table 5) without affecting growth rate (data not shown), indicating that high levels of cross-linking may cause bile sensitivity. We thus tentatively conclude that low cross-linking or absence of cross-linking may be compatible with bile resistance while high levels of cross-linking may cause bile sensitivity.

PG remodelling may be a common phenomenon in the prokaryotic world. Bacterial species that undergo

developmental processes are known to reshape their cell wall (Takacs *et al*., 2009; Morlot *et al*., 2010). In other cases, PG remodelling may be part of adaptive responses. For instance, *S. enterica* remodels PG in response to environmental cues other than the presence of bile salts (Kenyon *et al*., 2007) and during growth within epithelial cells (Quintela *et al*., 1997). Other intracellular pathogens that undergo PG remodelling are *Listeria monocytogenes* (Garcia-Del Portillo and Pucciarelli, 2012) and *Mycobacterium tuberculosis* (Both *et al*., 2011). In pathogens that colonise mucosal surfaces, PG remodelling contributes to lysozyme resistance (Davis and Weiser, 2011). In *Vibrio cholerae* and *Bacillus subtilis*, incorporation of D-amino acids into PG may be an adaptation to stationary phase physiology (Lam *et al*., 2009).

Even though barriers provided by the bacterial envelope reduce bile salt uptake, bile resistance requires the concurrence of additional mechanisms. Bile salts can enter the cell by diffusion, and active efflux is necessary to reduce their concentration inside the cell (Thanassi *et al*., 1997). At the same time, stress responses need to be induced to cope with the damage caused by bile salts to membranes (Begley *et al*., 2005; Merritt and Donaldson, 2009), to DNA (Prieto *et al*., 2004; 2006) and to the intracellular protein pool (Begley *et al*., 2005). Despite these challenges, *Salmonella* is able to thrive in the hepatobiliary tract and can remain for a lifetime in the harsh, inhospitable environment of the gall bladder (Gonzalez-Escobedo *et al*., 2011), a remarkable example of bacterial adaptation, among many others (Baumler *et al*., 2011; Casadesus, 2012).

Experimental procedures

Bacterial strains, bacteriophages, and standard strain construction

All the strains of *S. enterica* listed in Supporting information Table S1 belong to serovar Typhimurium, and derived from the mouse-virulent strain SL1344. For conciseness, *S. enterica* serovar Typhimurium is routinely abbreviated as *S. enterica*. Transduction was performed with phage P22 HT 105/1 *int201* (Schmieger, 1972; G. Roberts, unpublished). The P22 HT transduction protocol was described elsewhere (Garzon *et al*., 1995). To obtain phage-free isolates, transductants were purified by streaking on green plates, prepared according to Chan and colleagues (1972), except that methyl blue (Sigma Chemical, St Louis, MI) substituted for aniline blue. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Oligonucleotides used in this study are listed in Supporting information Table S2. Targeted gene disruption was achieved using plasmids pKD13 (Datsenko and Wanner, 2000) and oligonucleotides FOR and REV. Oligonucleotides E1 and E2 were used for allele verification. Antibiotic resistance cassettes introduced during strain construction were excised by recombination with plasmid pCP20 (Datsenko and Wanner, 2000).

Plasmids p_{BAD}-*ybiS* (pIZ2018) and p_{BAD}-*ynhG* (pIZ2019) were constructed by cloning the respective genes under the control of the arabinose-dependent p_{BAD} promoter of pBAD18 (Guzman *et al*., 1995) using the pairs of oligonucleotides ybiS-pBAD-FOR and ybiS-pBAD-REV, and ynhG-pBAD-FOR and ynhG-pBAD-REV respectively (Supporting information Table S2).

Growth media and conditions

LB broth was used as liquid medium. Liquid cultures were grown with aeration by shaking in an orbital incubator. If necessary for plasmid maintenance or selection of recombinant strains appropriate antibiotics, kanamycin sulfate (Km) or ampicillin (Ap), were added to broth or agar plates at a final concentration of $50\ \mu g\ ml^{-1}$ and $100\ \mu g\ ml^{-1}$ respectively. When specified, DOC (Sigma Chemical) was added. Cultures in the presence of DOC were started with an aliquot from an overnight grown culture in LB. Solid LB contained agar at 1.5% final concentration. To induce the arabinose-dependent promoter of pBAD18, 0.2% of arabinose was added.

Determination of minimal inhibitory concentrations

Exponential cultures in LB broth were prepared, and samples containing around 3×10^2 CFUs were transferred to polypropylene microtiter plates (Soria Genlab, Valdemoro, Spain) containing known amounts of DOC. After 24 h incubation at 37°C, growth was visually monitored. Assays were carried out in triplicate. Student's *t*-test was used to analyse every MIC. The null hypothesis was that MICs were not significantly different from the MIC for the wild-type. *P* values of 0.01 or less were considered significant.

Viability assays

Aliquots from *S. enterica* exponential cultures grown in LB, each containing around 2×10^6 cells, were treated with 9% and 11% of DOC for 30 min. The cultures were then diluted in 0.9% NaCl, plated on LB and incubated overnight at 37°C. CFUs were then counted. Assays were carried out in triplicate.

Subcellular fractionation and Western blotting

Fractions enriched in PG, inner membrane and outer membrane were obtained as described (Pucciarelli *et al*., 2002; Pucciarelli and Garcia-del Portillo, 2003). Bacteria were grown with shaking at 37°C in LB, LB + DOC 5% and LB + DOC 11% up to saturation (OD_{600} ~3 for LB cultures and ~2 for cultures with DOC). The volume of fractions loaded onto the gel was adjusted to the following numbers of bacteria: 2×10^8 (peptidoglycan, PG fraction) or 2×10^7 (for inner membrane, IM fraction; and outer membrane, OM fraction). Western blotting was performed as described (Pucciarelli and Garcia-del Portillo, 2003) using rabbit polyclonal anti-Lpp, anti-OmpA and anti-Pal immune sera at 1:10,000 dilution. The secondary antibody used was goat anti-rabbit conjugated to horseradish peroxidase (BioRad).

Peptidoglycan purification and muramidase digestion

PG was prepared as described before (Gonzalez-Leiza *et al*., 2011). Cells from exponential or stationary cultures (25 ml) were harvested by centrifugation and re-suspended in one volume of the culture medium used. The samples were slowly dropped into an equal volumen of boiling 10% (wt/vol) SDS and vigorously stirred for more than 4 h and left stirring overnight at 37°C. The insoluble fraction (PG) was recovered by centrifugation (300,000× *g*, 15 min, 30°C) and washed until it was free of SDS by successive suspension in distilled water and high speed centrifugation. The pellet was suspended in 20 mM Tris-HCl (pH 7.5) and digested first with 100 μg ml^{-1} α-amylase (EC 3.2.1.1; Sigma-Aldrich, Saint Louis, MO) at 37°C for 1 h, and then with 100 μg ml^{-1} preactivated pronase E (EC 3.4.24.4; Merck, Darmstadt, Germany) at 60°C for 90 min. The enzymes were inactivated by boiling for 2 min in 1% (final concentration) SDS. Cell walls were collected by centrifugation as described above, washed with water and centrifuged to remove insoluble debris. The pellet was digested in 50 mM phosphate buffer (pH 4.9) with Cellosyl (muramidase; Hoechst AG, Frankfurt, Germany) 100 μg ml^{-1} final concentration. Digestion was allowed to proceed overnight at 37°C and stopped by boiling the sample for 2 min in a water bath. The preparation was centrifuged at an Eppendorf centrifuge at maximum speed for 10 min. The supernatant (muropeptides) was mixed with one-third volume of 0.75 M sodium borate buffer (pH 9.0) and reduced with excess sodium borohydride ($NaBH_4$) for 30 min at room temperature. The pH was tested with pH indicator strips (Acilit, Merck) and adjusted to 3 with orthophosphoric acid. The samples were stored at −20°C.

Separation and quantification of muropeptides

Separation of the reduced muropeptides by HPLC (325 system; Kontron Instruments) was performed by the method of Glauner (1988). The eluted muropeptides were monitored by measuring absorbance at 204 nm (Jasco UV-1570 spectrophotometer). Individual muropeptides were quantified from their integrated areas using samples of known concentration as standards (Work, 1957). Muropeptide composition was determined by HPLC. Quantification of each muropeptide species was performed by integration of the peaks of the HPLC profile, and the muropeptides were grouped into classes according to structural similarities (Glauner, 1988). Chromatograms are presented as supplementary materials (Supporting information Figs S1–S7).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. HPLC separation of muropeptides from exponential cultures of *S. enterica* SL1344 grown in LB (blue) and LB supplemented with 5% DOC (red).
Fig. S2. HPLC separation of muropeptides from stationary cultures of *S. enterica* SL1344 grown in LB (blue) and LB supplemented with 5% DOC (red).
Fig. S3. HPLC separation of muropeptides from stationary cultures of. *S. enterica* SL1344 grown in LB (blue) and LB supplemented with 11% DOC (red).
Fig. S4. HPLC separation of muropeptides from stationary cultures of *S. enterica* strains SV7756 (Δ*ybiS* Δ*erf* Δ*ycfS*/p_{BAD}::*ybiS*) and SV7753 ((Δ*ybiS* Δ*erf* Δ*ycfS*/pBAD) grown in LB.
Fig. S5. HPLC separation of muropeptides from stationary cultures of *S. enterica* strains SV7755 (SL1344/p_{BAD}::*ynhG*) and SV7464 (SL1344/pBAD) grown in LB.
Fig. S6. HPLC separation of muropeptides from stationary cultures of. *S. enterica* strains SV7757 (Δ*ycbB* Δ*ynhG*/p_{BAD}::*ybiS*) and SV7753 Δ*ycbB* Δ*ynhG*/p_{BAD}).
Fig. S7. HPLC separation of muropeptides from stationary cultures of *S. enterica* strains SV7760 (Δ*ybiS* Δ*erf* Δ*ycfS*/p_{BAD}::*ynhG*) and SV7761 (Δ*ybiS* Δ*erf* Δ*ycfS*/pBAD) grown in LB.
Table S1. Strains of *Salmonella enterica* serovar Typhimurium constructed for this study.
Table S2. Oligonucleotides used in this study.