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A novel peptidoglycan D,L-endopeptidase induced by *Salmonella* inside eukaryotic cells contributes to virulence

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Summary

Bacteria remodel peptidoglycan structure in response to environmental changes. Many enzymes are involved in peptidoglycan metabolism; however, little is known about their responsiveness in a defined environment or the modes they assist bacteria to adapt to new niches. Here, we focused in peptidoglycan enzymes that intracellular bacterial pathogens use inside eukaryotic cells. We identified a peptidoglycan enzyme induced by *Salmonella enterica* serovar Typhimurium in fibroblasts and epithelial cells. This enzyme, which shows γ-D-glutamyl-*meso*-diaminopimelic acid D,L-endopeptidase activity, is also produced by the pathogen in media with limited nutrients and in resting conditions. The enzyme, termed EcgA for endopeptidase responding to cessation of growth', is encoded in a *S.* Typhimurium genomic island absent in *Escherichia coli*. EcgA production is strictly dependent on the virulence regulator PhoP in extra- and intracellular environments. Consistent to this regulation, a mutant lacking EcgA is attenuated in the mouse typhoid model. These findings suggest that specialised peptidoglycan enzymes, such as EcgA, might facilitate *Salmonella* adaptation to the intracellular lifestyle. Moreover, they indicate that readjustment of peptidoglycan metabolism inside the eukaryotic cell is essential for host colonisation.

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Introduction

The peptidoglycan or murein is a macromolecular polymer composed by glycan chains of alternating N-acetyl-glucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues linked by ß(1-4) glycosidic bonds. Stem peptides bound to the MurNAc moiety cross-link the glycan chains resulting in the formation of a macromolecular lattice (Vollmer *et al.*, 2008; Cava *et al.*, 2013). A remarkable feature of the peptidoglycan is its structural plasticity. Bacteria manifest structural changes in this molecule during growth transitions or adaption to new environmental niches (Vollmer *et al.*, 2008; Alvarez *et al.*, 2014). Early studies in *Bacillus subtilis* showed increase in cross-linked muropeptides when vegetative cells transit from an active state of growth to stationary phase (Atrih *et al.*, 1999). *Escherichia coli* cells in stationary phase reduce length of glycan chains and, in addition, increase the percentage of muropeptides covalently bound to Braun's lipoprotein (Pisabarro *et al.*, 1985). During nutritional stress, *Vibrio cholerae* incorporates non-canonical D-amino acids in the peptidoglycan (Lam *et al.*, 2009). These D-amino acids affect peptidoglycan composition, strength and relative amount of the macromolecule per cell. Peptidoglycan structure also changes at a large extent during sporulation. Unlike in vegetative cells, the peptidoglycan of the cortex is enriched in muropeptides lacking stem peptides and muropeptides bearing a single L-Ala residue bound to the MurNAc moiety (Atrih and Foster, 2001). Peptidoglycan structure is altered by the pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) within epithelial cells. Intracellular *S.* Typhimurium builds a peptidoglycan in which a large proportion of stem peptides in cross-linked muropeptides are trimmed by the action of an MurNAc-L-Ala amidase (Quintela *et al.*, 1997).

Peptidoglycan modification in bacterial pathogens is important for host colonisation (Humann and Lenz, 2009; Davis and Weiser, 2011). Thus, many peptidoglycan structural changes described in pathogens impair recognition by host degradative enzymes such as lysozyme, which cleaves the glycan chain (Humann and Lenz, 2009; Davis and Weiser, 2011). Modifications in L-Ala, D-Glu, or *meso*-

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dap/lysine residues of the stem peptide diminish the effectiveness of host intracellular defence receptors such as Nod1 and Nod2, which sense muramyl-peptides and stem peptides having defined motifs (Humann and Lenz, 2009; Philpott *et al*., 2014). In some cases, peptidoglycan modification has the reverse effect. Thus, deacetylation of sugar residues by the enzyme PgdA stimulates *Helicobacter pylori* recognition by Nod1 (Suarez *et al*., 2015). The impact of these modifications in virulence was uncovered after studying mutants lacking the candidate modifying enzymes. Examples are *Listeria monocytogenes*, *Staphyloccocus aureus* and *H. pylori* mutants that do not modify the sugars of the glycan chains and, as a consequence, display altered virulence phenotypes (Bera *et al*., 2006; Boneca *et al*., 2007; Aubry *et al*., 2011; Wang *et al*., 2012; Burke *et al*., 2014). The infection phase and the host location where these peptidoglycan-modifying enzymes are produced by the pathogen were, however, not determined in most of these studies. One of the few exceptions is the enzyme PgdA of *Streptococcus suis*, which was shown to contribute to virulence and to be induced *in vivo* (Fittipaldi *et al*., 2008).

Despite the large number of studies that have analysed the biochemistry of peptidoglycan-modifying enzymes in bacterial pathogens, little is yet known about the regulation of these enzymes during the infection process. In this work, we characterised an *S*. Typhimurium enzyme that shows hydrolytic activity on purified muropeptides and is encoded by a gene absent in *E. coli*. Novel features found for this enzyme are its induction in bacteria located inside eukaryotic cells and its strict control by the virulence regulator PhoP, which plays a master role in the adaptation of *S*. Typhimurium to the intracellular lifestyle.

Results

S*. Typhimurium harbours a genomic island encoding a putative cell wall hydrolase*

S. Typhimurium proliferation within HeLa epithelial cells occurs concomitantly to a profound peptidoglycan remodelling process that is not observed in bacteria grown in laboratory media. Thus, the peptidoglycan of intracellular proliferating bacteria contains increased amounts of cross-linked dimeric muropeptides lacking one of the two GlcNAc-MurNAc disaccharide moieties (Quintela *et al*., 1997). We reasoned that novel enzymes responding to environmental cues of the eukaryotic cell could be responsible for such unique structural changes. To test this idea, we mined the genome of *S*. Typhimurium virulent strain SL1344 for uncharacterised genes encoding predicted proteins related to cell wall metabolism. Among the candidate genes, we prioritised those: (i) showing a restricted phylogenetic distribution, i.e. absent in non-pathogenic bacteria; and (ii) upregulated by intracellular *S*. Typhimurium according to our recent transcriptome analyses (Núñez-Hernández *et al*., 2013). We identified *SL1344_1873*, a gene annotated as encoding a ‘putative cell wall hydrolase’ that bears an NlpC_P60 endopeptidase domain. *SL1344_1873* shows increased expression in non-growing intracellular bacteria (Núñez-Hernández *et al*., 2013) and is orthologue to the gene *STM1940* of LT2 strain, which is genome reference for the *S*. Typhimurium serovar (McClelland *et al*., 2001). BLASTp searches showed that the SL1344_1873 protein is not encoded in the genome of most *Escherichia coli* strains with the exception of the environmental isolate TW09276 (57% identity over 99% coverage of SL1344_1873, E-value = 0.0) (Luo *et al*., 2011) and KTE52, a human isolate (56% identity over 99% coverage of SL1344_1873, E-value = 0.0). In *S*. Typhimurium strain SL1344, *SL1344_1873* is flanked by *SL1344_1872* and *SL1344_1874*, two genes of unknown function that are absent in *E. coli*, as revealed by BLASTn searches. Outwards to *SL1344_1872* and *SL1344_1874*, the *S*. Typhimurium SL1344 genome contains *yecA*, a gene of unknown function, and a pseudogene (*SL1344_1874A*) that is followed by the tRNA-encoding genes *leuZ*, *cysT* and *glyW* (Fig. 1). *yecA* and these three tRNA-encoding genes are conserved in *S*. Typhimurium and *E. coli* (Fig. 1). BLASTn searches confirmed that the genetic configuration encompassing *yecA – SL1344_1872 – SL1344_1873 – SL1344_1874 – SL1344_1874A – leuZ – cysT – glyW* is found exclusively in *Salmonella*. Based on this, we concluded that the region *SL1344_1872 – SL1344_1873 – SL1344_1874 – SL1344_1874A* is a genomic island. The low G + C content of these four genes (42.8%, 48.5%, 36.9% and 47.7%, respectively) (Fig. 1) compared with the average 52% G + C of the *Salmonella* genome supports their acquisition by horizontal transfer. Moreover, the low G + C content (36.9%) of *SL1344_1874* suggests that it could have been incorporated into the genome in a separate event. Such predicted modular evolution of this island is denoted in the genome of the non-pathogenic species *S. bongori*, which harbours homologues to the *S*. Typhimurium genome island except part of *SL1344_1874* and the *SL1344_1874A* pseudogene (Fig. 1).

BLASTp searches outside the *Salmonella* and *Escherichia* genera detected some putative orthologues of SL1344_1872, SL1344_1873 and SL1344_1874 with identities in the 30–63% range and 80–100% query coverage. None of these three proteins, however, show common phylogeny (Fig. S1), consistent with a genomic island built exclusively in *Salmonella* in several unrelated events. Altogether, these analyses showed that the *SL1344_1873* gene, encoding a putative cell wall hydrolase and induced by intracellular bacteria, maps in a new *Salmonella* genomic island.

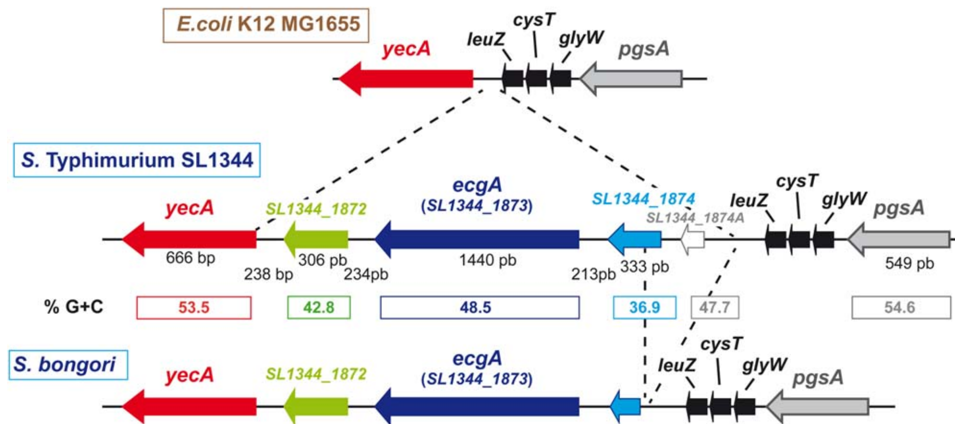

Fig. 1. *SL1344_1873* (*ecgA*) maps in a *S. enterica* genomic island that is absent in most *E. coli* strains. Schematic view of the *S.* Typhimurium genome region harbouring *SL1344_1873* (*ecgA*). This gene is conserved in all *S. enterica* serotypes and absent in most *E. coli* strains (see text). Flanking genes *SL1344_1872* and *SL1344_1874* show the same phylogenetic distribution as *SL1344_1873* (*ecgA*). Indicated are the genome regions of *E. coli* K12 strain MG1655 (accession NC_000913.3), *S. enterica* serovar Typhimurium strain SL1344 (accession NC_016810.1) and *S. bongori* strain N268-08 (accession NC_021870.1). Note that the *S. bongori* genome lacks a fragment of the island upstream to *SL1344_1873* (*ecgA*). This region encompasses part of *SL1344_1874* and the pseudogene *SL1344_1874A*. The G + C content for each of the genes shown is indicated for the case of the *S.* Typhimurium *SL1344* genome. *leuZ*, *cysT* and *glyW* are tRNA-encoding genes.

S. *Typhimurium produces the* SL1344_1873 *protein in growth cessation conditions and inside eukaryotic cells*

To determine growth conditions that favour production of the SL1344_1873 protein, we generated a strain bearing a 3×FLAG-tagged allele in its native chromosomal location. Bacteria were grown in nutrient rich LB medium and in minimal media as intracellular salt medium (ISM) or PCN (phosphate-carbon-nitrogen limiting medium). PCN was acidified to pH 5.8, a condition that stimulates expression of virulence factors produced by *S.* Typhimurium inside eukaryotic cells (Deiwick *et al*., 1999). Western blot assays showed that *S.* Typhimurium produces SL1344_1873 following growth cessation in LB (Fig. 2A) and in acidified PCN medium (Fig. 2A). SL1344_1873 is also produced in ISM medium, regardless of the growth state of the bacteria (Fig. 2A). SL1344_1873 production therefore associates to either growth arrest or proliferation in a limited nutrient medium that mimics the intracellular environment of eukaryotic cells. Based on this expression profile, we tested whether bacteria that are in a non-proliferative state inside fibroblasts produce SL1344_1873 (Garcia-del Portillo *et al*., 2008). Western blot assays showed higher SL1344_1873 protein levels in non-growing intracellular bacteria compared with the extracellular bacteria that were used to infect the eukaryotic cells (Fig. 2B). Intracellular bacteria located in HeLa epithelial cells also showed increased SL1344_1873 protein levels (Fig. 2B). These data demonstrated that *S.* Typhimurium upregulates SL1344_1873 expression inside eukaryotic cells. This expression profile is also consistent with the abundance of this protein in bacteria that cease growth or cope with limited nutrients. Based on these findings, we renamed SL1344_1873 as EcgA, for 'putative endopeptidase responding to cessation of growth'.

EcgA expression is regulated by the PhoP-PhoQ two-component system

The two-component regulatory system PhoP-PhoQ controls many functions expressed by *S.* Typhimurium inside distinct eukaryotic cell types such as macrophages and fibroblasts (Cano *et al*., 2001; Kato and Groisman, 2008; Prost and Miller, 2008; Núñez-Hernández *et al*., 2013). As revealed by Western blot assays, *phoP* mutant bacteria isolated from fibroblasts do not produce EcgA (Fig. 2B). EcgA production was also abolished in *phoP* mutant bacteria growing in LB, acidified PCN or ISM media (Fig. 2C). Consistent with this strict requirement of PhoP for *ecgA* expression, wild-type bacteria did not express EcgA when incubated in a medium containing high concentration of magnesium, 10 mM Mg^{2+} (Fig. 2D), a condition known to repress PhoP-PhoQ (Garcia Vescovi *et al*., 1996). Taken together, these data demonstrated that PhoP regulates positively *ecgA* expression in extra- and intracellular environments.

ecgA *expression depends on a PhoP-regulated promoter located in the upstream gene* SL1344_1874

EcgA protein levels are severely reduced in a *phoP* background (Fig. 2), which led us to explore the underlying regulatory mechanism. To this aim, we replaced the complete *ecgA* coding region by a *lacZ* reporter (Fig. 3A). When bacteria were incubated in 10 mM Mg^{2+}, *ecgA*::*lacZ* expression diminished in wild-type bacteria to the levels

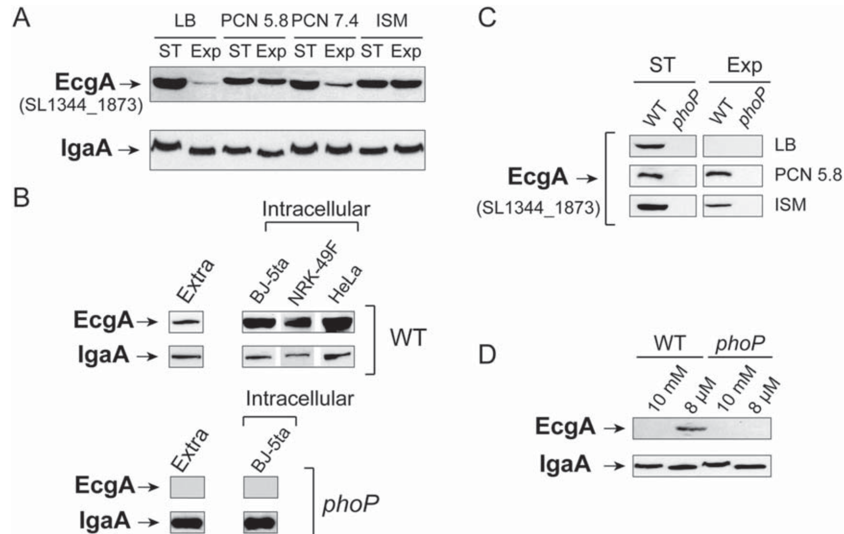

Fig. 2. EcgA (SL1344_1873) production is favoured in non-growing or nutrient limiting conditions, inside eukaryotic cells and is regulated by PhoP.
A. EcgA protein levels detected by Western in total protein extracts obtained from a *S.* Typhimurium *ecgA*::3×FLAG tagged strain grown in the indicated media and growth conditions. ST, stationary phase; Exp, exponential phase.
B. EcgA production is enhanced by *S.* Typhimurium inside fibroblasts (BJ-5ta, NRK-49F) and epithelial cells (HeLa). EcgA production is abolished in *phoP* mutant that overgrows inside fibroblasts.
C. Western blots showing positive regulation of EcgA by PhoP in LB, PCN pH 5.8, and ISM media. ST, stationary phase; Exp, exponential phase.
D. EcgA expression is repressed by high Mg^{2+} in wild-type bacteria. Minimal N medium containing low (8 μM) or high (10 mM) $MgCl_2$ concentration was used. Membrane protein IgaA was detected as loading control. Data are representative of a minimum of three independent experiments.

detected in a *phoP* background (Fig. 3B). PhoP-PhoQ therefore regulates *ecgA* expression at the transcriptional level.

Recent RNA-sequencing data show a transcription start site (TSS) 19 nt downstream the annotated start ATG codon of the upstream gene *SL344_1874*, concretely in the coordinate 1,992,661 of the strain SL1344 genome (Kröger *et al*., 2013). We cloned in front of a promoter-less *lacZ* gene a 197 nt fragment that encompasses such TSS and ends 29 nt downstream this site. This reporter construct, referred as pProm-*SL1344_1874*::*lacZ* (Fig. 3C), was maximally expressed in the absence of Mg^{2+} and repressed in either high Mg^{2+} concentration (10 mM Mg^{2+}) or in a *phoP* background (Fig. 3D). MEME/MAST (http://meme-suite.org/) analysis predicted a PhoP-binding site 50 nt upstream the TSS and in reverse orientation (Fig. 3E). Electrophoresis mobility shift assays showed a band shift that increased in proportion when PhoP was phosphorylated *in vitro* with acetyl-phosphate (Fig. 3F). Requirement of the PhoP-binding box for pProm-*SL1344_1874*::*lacZ* expression was confirmed after introducing two different mutations (Mut1 and Mut2) in this regulatory site (Fig. 3E). Both mutations abolished reporter gene expression (Fig. 3D). Of interest, PhoP-dependent expression of *ecgA* was abrogated by a polar Cm^R cassette inserted in reverse orientation 32 bp downstream the described TSS (Fig. 3A and B). Taken together, these data demonstrated that a PhoP-dependent promoter located within *SL344_1874* controls *ecgA* expression.

As abovementioned, the TSS of *ecgA* is located 19 bp downstream the ATG translational start site predicted for *SL344_1874* (Kröger *et al*., 2013). Using a strain harbouring a *SL344_1874*::3xFLAG allele tagged at the 3′ end, we were able to detect by Western a 10.6 kDa protein whose production is also PhoP-dependent (data not shown). This protein size, lower than the theoretical 12.4 kDa when considering the predicted ATG starting codon, suggested an alternative translational site for *SL344_1874*. Consistently, 29 bp downstream the TSS there is a GTG codon that could act as translational start site for a slightly shorter version of SL344_1874 protein. Insertion of a *lacZ* reporter gene immediately downstream this GTG codon showed similar diminished expression in either 10 mM Mg^{2+} or a *phoP* background (Fig. 3B). These findings demonstrated that the PhoP-regulated promoter drives concomitantly expression of both *SL344_1874* and *ecgA (SL344_1873)*.

EcgA *is a peptidoglycan D,L-endopeptidase that cleaves the γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) bond in uncross-linked muropeptides*

ecgA encodes a predicted 479 amino acid putative ‘cell wall hydrolase’ with a four-domain architecture. These four domains include: (i) an N_NlpC_P60 stabilising domain

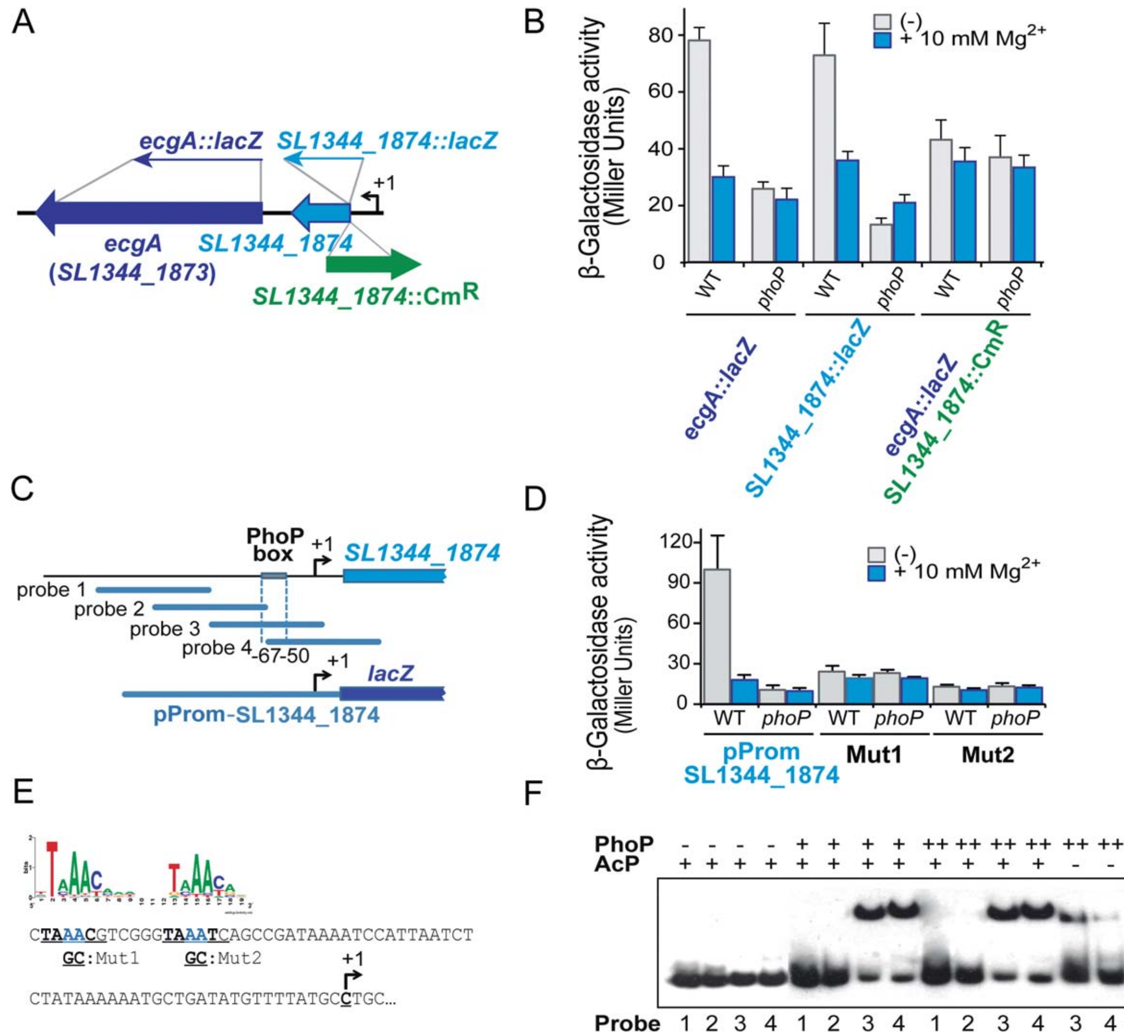

Fig. 3. The *SL1344_1874-ecgA* operon is under PhoP transcriptional control.
A. Genetic organisation of the *SL1344_1874-ecgA* operon indicating the *lacZ* reporter gene and the chloramphenicol (Cam) resistance cassette insertions.
B. β-Galactosidase activity measured from *ecgA*::*lacZ*, *SL344_1874*::*lacZ* and *ecgA*::*lacZ SL344_1874*::Cam transcriptional fusions expressed in wild-type or *phoP* genetic backgrounds. Bacteria were grown overnight in LB without (–) or with 10 mM Mg^{2+}.
C. Scheme of the *SL1344_1874* promoter controlling the expression of the *SL1344_1874-ecgA* operon. The PhoP box, the promoter probes used for the EMSA assay and the DNA fragment cloned in pProm-SL1344_1874 are shown.
D. β-galactosidase activity derived from the native, the Mut1 and the Mu2 p*SL1344_1874*-derived promoters in cells grown overnight in LB without (–) or with 10 mM Mg^{2+}.
E. DNA sequence of the p*SL1344_1874* promoter. The transcriptional start site and the direct repeats recognised by PhoP are in bold and underlined. Mutations introduced to disrupt the PhoP recognition (Mut1 and Mut2) are also indicated.
F. EMSA was performed using a 5′-^{32}P-end-labelled PCR probes indicated in (C) incubated with 1 μM (+) or 2 μM (++) of PhoP-6×His in the absence (–) or presence (+) of acetyl-phosphate, AcP. Data shown in panels (B) and (D) correspond to mean values of at least four independent experiments performed in duplicate. Error bars correspond to the standard deviations.

(Pfam accession PF12912) located at the N-terminus; (ii) a bacterial SH3_6 domain (Pfam PF12913); (ii) a bacterial SH3_7 domain (Pfam PF12914); and (iv) a C-terminal NlpC_P60 catalytic domain (Pfam PF00877) of the cysteine-peptidase type. This later NlpC_P60 catalytic domain is present in a large family of peptidoglycan endopeptidases (Anantharaman and Aravind, 2003). The TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) did not detect any trans-membrane region in EcgA, and the SignalP v4.1 tool (http://www.cbs.dtu.dk/services/SignalP/) predicted a signal peptide with a cleavage site between residues 21–22. These features indicated that EcgA could be located in the periplasm. Western assays confirmed the presence of EcgA in the membrane fraction (Fig. 4A), consistent with a probable peptidoglycan hydrolytic activity. The predicted endopeptidase activity was examined *in vitro* using purified EcgA and, as substrates, uncross-linked (monomeric) or cross-

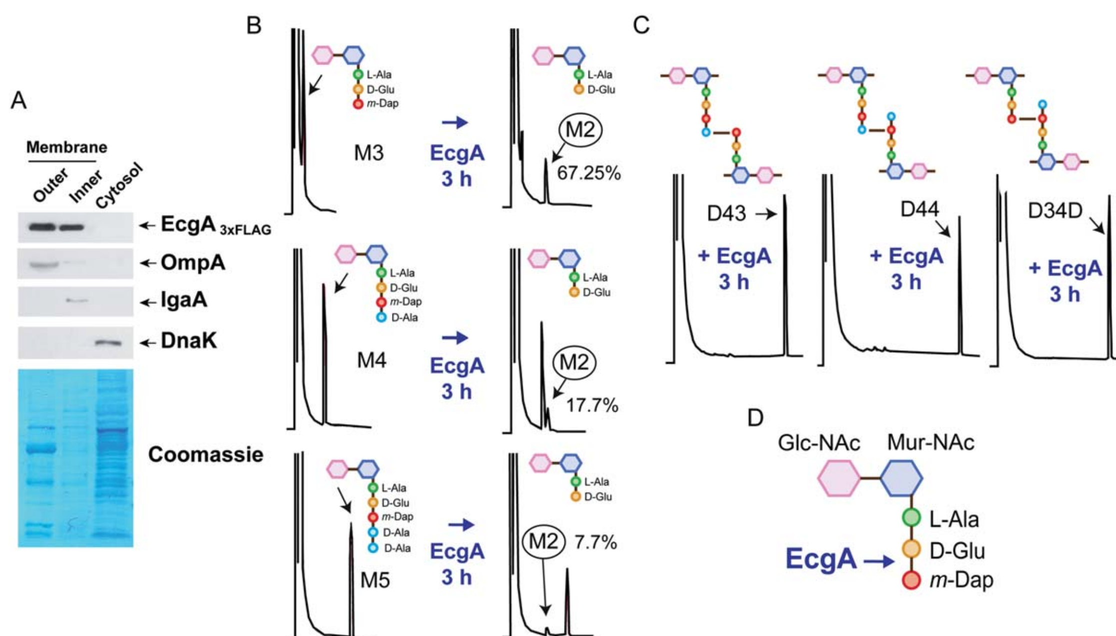

Fig. 4. EcgA (SL1344_1873) is a membrane-associated D,L-endopeptidase with activity on the γ-D-isoglutamic-*meso*-dap (iE-DAP) bond.
A. Western blot of subcellular fractions showing that EcgA is enriched in membrane (outer and inner) fractions. OmpA, IgaA and DnaK were used as controls of outer-, inner membrane and cytosol respectively.
B. Purified EcgA (EcgA′-6×His tagged variant) cleaves monomeric muropeptides M3 (GlcNAc-MurNAc-L-Ala-D-Glu-dap); M4 (GlcNAc-MurNAc-L-Ala-D-Glu-dap-D-Ala); and M5 (GlcNAc-MurNAc-L-Ala-D-Glu-dap-D-Ala-D-Ala). In all cases, M2 (GlcNAc-MurNAc-L-Ala-D-Glu) was obtained as product. The percentage of M2 versus total amount of muropeptide is indicated for each reaction. Shown are the HPLC profiles of the muropeptide at time 0 and after 3 h of incubation with the enzyme.
C. Lack of endopeptidase activity of EcgA on dimeric muropeptides. Shown are the HPLC profiles obtained after the 3 h of incubation with the enzyme.
D. γ-D-isoglutamic-*meso*-dap bond proposed as target for the D,L-endopeptidase activity of EcgA.

linked (dimeric) muropeptides bearing distinct stem peptides. Monomeric muropeptides included: M3 (GlcNAc-MurNAc-L-Ala-D-Glu-dap); M4 (GlcNAc-MurNAc-L-Ala-D-Glu-dap-D-Ala); and M5 (GlcNAc-MurNAc-L-Ala-D-Glu-dap-D-Ala-D-Ala). Incubation of these muropeptides with EcgA resulted in all cases in M2 (GlcNAc-MurNAc-L-Ala-D-Glu) as product of the reaction (Fig. 4B). The reaction efficiency was strongly influenced by the length of the stem peptide. M3 was the preferred substrate (65% converted into M2 after 3 h incubation) followed by M4 and M5 (17.7 and 7.7% conversion, respectively) (Fig. 4B). Purified EcgA did not cleave cross-linked dimeric muropeptides (Fig. 4C), irrespective of their stereochemistry. These data showed that EcgA is a peptidoglycan D,L-endopeptidase (Fig. 4D) with activity on the γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) bond of uncross-linked muropeptides. EcgA could also be classified as a D,L-carboxypeptidase, as it can cleave the last amino acid of the stem peptide as it is occurs with M3.

EcgA *contributes to* S. *Typhimurium virulence*

We next used infection models to test whether EcgA could contribute to virulence. A Δ*ecgA* null mutant behaved as wild-type bacteria *in vitro* in both the persistent infection model of fibroblasts and the active proliferative state displayed by this pathogen within epithelial cells (Fig. 5A). A slight but reproducible higher load of intracellular Δ*ecgA* mutant compared with wild-type bacteria was however observed in cultured fibroblasts at all post-infection times: 2, 24 and 48 h (Fig. 5B). No such difference in intracellular bacterial load was noted in epithelial cells (Fig. 5B). Competition experiments in BALB/c mice showed that EcgA is required for wild-type bacteria to efficiently colonise the liver and spleen. This attenuation was evident in bacteria administrated by the intra-peritoneal route (Fig. 5C). Virulence assays with complemented strains were not possible as expression of EcgA from a plasmid impaired bacterial growth (Fig. S2). This deleterious effect could probably be the result of expressing in actively growing bacteria a hydrolytic enzyme which is normally produced in resting or slow growth conditions (Fig. 2). Generation of a complemented strain harbouring in the chromosome a wild-type *ecgA* allele with its own promoter was not attempted, mainly due to the complex regulatory scheme involving the upstream gene *SL1344_1874* and the PhoP-dependent promoter shared by these two genes (Fig. 3). Importantly, RT-PCR experiments showed that

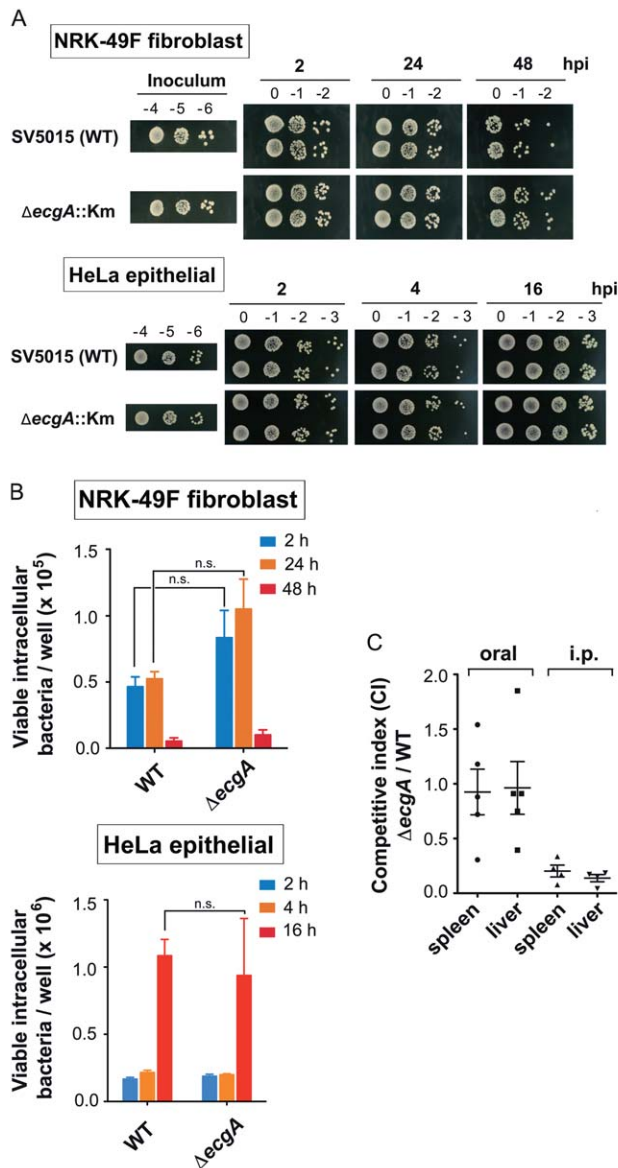

Fig. 5. EcgA (SL1344_1873) function modulates intracellular bacterial load and is required for virulence in BALB/c mice.
A. Load of wild-type and Δ*ecgA* bacteria estimated in samples obtained from extracellular cultures (inoculum) and compared with intracellular bacteria present in lysates of NRK-49F fibroblasts at 2, 24 and 48 h post-infection and HeLa epithelial cells at 2, 4 and 16 h post-infection.
B. Intracellular bacteria quantified in NRK-49F fibroblasts and HeLa epithelial cells infected with wild-type and Δ*ecgA* isogenic strains. Note the slight higher number of viable intracellular bacteria registered in fibroblasts for the Δ*ecgA* mutant at all post-infection times. Data are the means and standard deviations from three independent experiments.
C. Lack of EcgA reduces bacterial fitness in BALB/c mice after intraperitoneal (i.p.) but not oral administration. Shown are the competitive index (C.I.) of Δ*ecgA* versus wild-type bacteria in both liver and spleen. Organ extracts were prepared at 5 days (oral challenge) or 48 h (i.p. challenge) after mouse infections.

the Δ*ecgA* null mutation tested in the virulence assays does not have any polar effect relative to the expression of the flanking genes *SL1344_1874* and *SL1344_1872* (Fig. S3). Taken together, the data obtained in the virulence assays demonstrated that EcgA is a peptidoglycan D,L-endopeptidase of *S*. Typhimurium that contributes to systemic infection in mice.

Discussion

To our knowledge, this study provides the first evidence at the protein level for the induction of a peptidoglycan enzyme in bacteria located inside eukaryotic cells. This enzyme, named EcgA, is a D,L-endopeptidase that cleaves the γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) bond in monomeric muramyl-peptides and contributes to the systemic infection of mice by *S*. Typhimurium. Our findings reinforce the view of peptidoglycan remodelling as a process essential for pathogens to succeed in the infection.

Many modifications involving loss or gain of specific chemical groups in the glycan chain or in the stem peptide confer resistance to host defences (Humann and Lenz, 2009; Sukhithasri *et al*., 2013). Considering the capacity of EcgA to cleave the iE-DAP bond, our study extends this view as pathogens could also succeed in the infection by actively diminishing the presence of motifs recognised by host defences. This idea fits well in the case of intracellular bacterial pathogens. Once these pathogens transit to and colonise the intracellular niche of the eukaryotic cell, they must avoid recognition by specialised receptors as those of the Nod family (Nod1 and Nod2), which bind muramyl-peptides (Caruso *et al*., 2014; Philpott *et al*., 2014). We hypothesise that *S*. Typhimurium induces EcgA inside eukaryotic cells to impair signalling by Nod1, which recognises muramyl-peptides bearing the iE-DAP motif. As EcgA action would produce muramyl-dipeptide, a ligand of Nod2, it is still possible that the infected fibroblast could recognise non-growing intracellular bacteria via this receptor. However, RT-PCR assays performed with RNA extracted from human and mouse fibroblasts indicate that this possibility is unlikely as *NOD2* is not expressed by these fibroblasts even following exposure to bacteria (data not shown). The EcgA-dependent evasion strategy we postulate for *S*. Typhimurium persisting inside the fibroblast has some parallelism to that described in *Legionella pneumophila*. Once inside macrophages, *L. pneumophila* uses the protein effector EnhC to inhibit function of Slt, a lytic transglycosylase (Liu *et al*., 2012). Avoidance of glycan chain cleavage by Slt could reduce release of muramyl-peptides from macromolecular peptidoglycan. Of note, we did not detect changes in the HPLC muropeptide profile when purified EcgA was incubated with whole sacculi (Fig. S4). This result suggests that EcgA could

target free muramyl-peptides bearing the iE-DAP motif that are released from the peptidoglycan by lytic transglycosylases. This mechanism differs from those involving *S.* Typhimurium protein effectors translocated by the pathogen to stimulate Nod1 and Nod2 by direct protein–protein interactions (Keestra *et al.*, 2011; 2013). Our work, therefore, suggests that intracellular *S.* Typhimurium may also manipulate Nod-signalling restricting the availability of inducing molecules derived from the peptidoglycan. It is also probable that mere structural changes in the peptidoglycan, involving glycan chain length or peptide cross-linking level, can influence the infection outcome. This idea is supported by a recent study showing that some penicillin-binding proteins of *Staphylococcus aureus* are dispensable for growth in the laboratory but required for pathogenicity (Reed *et al.*, 2015).

EcgA is encoded by a genomic island composed of three genes and a pseudogene. Most peptidoglycan hydrolases acquired by horizontal transfer are muramidases encoded in prophages, phage remnants or functionally linked to pathogenicity islands that encode type III and type IV protein secretion systems (Zahrl *et al.*, 2005). Neither EcgA nor the products of the two flanking genes show similarity to known peptidoglycan enzymes of phages or protein secretion systems. The genomic island containing *ecgA* could have been specialised in peptidoglycan modification and, probably, have contributed to the evolution of *S.* Typhimurium as intracellular pathogen. Future work should discern whether some of the two flanking genes, both of unknown function, assist EcgA role as a D,L-endopeptidase. Coexpression of *SL1344_1874* and *ecgA* argues in favour of these two genes having a related function.

An interesting feature found for EcgA was its induction in conditions associated to cessation of active growth (stationary phase), persistence inside eukaryotic cells, and growth in media with limiting nutrients. EcgA might have evolved as a hydrolase that acts preferentially in response to nutritional stress. Under this situation, peptidoglycan recycling, which uses free muropeptides released in the periplasm as a result of lytic transglycosylases, was proposed to have a survival value (Park and Uehara, 2008). EcgA might modulate the recycling pathway, for which it has been shown that anhydro-M3 [GlcNAc-(anh)MurNAc-L-Ala-D-Glu-dap] is incorporated into the cytosol by the permease AmpG. Remarkably, no study has investigated whether 'anhydro-M2', which could derive from EcgA activity in the periplasm, might be also an AmpG substrate. As AmpG incorporates the disaccharide GlcNAc-(anh)MurNAc and anhydro-M3, there is no obvious reason to discard entry of 'anhydro-M2' into the recycling pathway.

Our data also revealed that EcgA is regulated by the major virulence regulator PhoP. In *S.* Typhimurium, PhoP controls expression of many horizontally acquired genes, including *pcgL*, that encodes a periplasmic D-Ala-D-Ala dipeptidase (Hilbert *et al.*, 1999). PcgL was reported to attenuate *S.* Typhimurium virulence (Mouslim *et al.*, 2002), a role contrasting with the requirement of EcgA to cause systemic disease. Contribution of EcgA to virulence agrees with the study that Chaudhuri *et al.* performed in different animal models (Chaudhuri *et al.*, 2013). These authors used pools of randomly mutagenised *S.* Typhimurium mutants to identify genes important for intestinal colonisation in calf, pig and chicken. Their data show that insertions in *ecgA* are negatively selected *in vivo* (Chaudhuri *et al.*, 2013). The reason for why in the mouse typhoid model EcgA is required for virulence when bacteria are administrated intraperitoneally and not by the oral route is, at present, unknown. Entry via these two routes differs regarding the host cell types that bacteria encounter in the first stages of the infection (Watson and Holden, 2010; Mastroeni and Grant, 2013), and this may affect intracellular survival of the mutant at different extent. Moreover, the mouse typhoid model is not comparable with those of calf, pig and chicken regarding level of inflammation during the course of the infection.

In summary, this work reports a novel peptidoglycan D,L-endopeptidase that is induced by *S.* Typhimurium inside eukaryotic cells and is controlled by a regulator essential for virulence. Our current aim is to dissect how EcgA activity on the peptidoglycan modulates the intracellular lifestyle of this pathogen in conditions in which its growth is restrained.

Experimental procedures

Bacterial strains, gene epitope tagging and growth conditions

S. Typhimurium strains used are isogenic to SV5015, a His$^+$ derivate of SL1344 (Hoiseth and Stocker, 1981). These strains include: MD3350 (Δ*ecgA*::Km); MD3351 (*ecgA*::3xFLAG-Km); MD3400 (*ecgA*::3xFLAG-Km *phoP7953*::Tn*10*); PB11352 (*ecgA*::*lacZ*); PB11361 (*ecgA*::*lacZ phoP7953*::Tn*10*); PB11355 (*SL344_1874*::*lacZ*); PB11362 (*SL344_1874*::*lacZ phoP7953*::Tn*10*); PB11353 (*ecgA*::*lacZ SL344_1874*::Cam); PB11363 (*ecgA*::*lacZ SL344_1874*::Cam *phoP7953*::Tn*10*). Genetic manipulation for gene inactivation and insertion of *lacZ* fusions were carried out using Lambda Red-mediated recombination (Ellermeier *et al.*, 2002). 3×FLAG epitope tagging was performed using the method of Uzzau *et al.* (2001). Oligonucleotides used are listed in Table S1. To clone the putative *STM1344_1874* TSS, a 197 nt fragment (coordinates 1,992,633 to 1,992,829 in SL1344 genome) was introduced in pMC1871 in front of a promoterless *lacZ* gene rendering pProm-SL1344_1874 plasmid. Mut1 and Mut2 promoter mutants (Fig. 3E) were generated by the megaprimer technique (Ibanez *et al.*, 2015). *E. coli* strain MD3761 (BL21(DE3)/pET22-*ecgA*::6×His) was used for EcgA purification (see below). Bacteria were routinely grown in at 37°C

Luria broth (LB). Growth in minimal media N (low 8 μM, or high 10 mM, Mg^{2+} concentration), PCN pH.5.8 and ISM was performed as described (Núñez-Hernández *et al.*, 2014). When appropriate, kanamycin (30 μg/ml), chloramphenicol (10 μg/ml), spectinomycin (100 μg/ml) or ampicillin (50 μg/ml) were added to the growth media. β-Galactosidase assays were carried out as described (Miller, 1972), using wild type, *phoP7953*::Tn*10* or Δ*phoPQ*::Sm strains (Aguirre *et al.*, 2006) grown overnight in LB without or with the addition of 10 mM $MgCl_2$.

Eukaryotic cell lines and bacterial infection assays

The fibroblast cell lines BJ-5ta (ATCC CRL-4001) and NRK-49F (ATCC CRL-1570) of human and rat origin, respectively, were used for infection assays. The epithelial cell line HeLa (ATCC CCL-2) was also used for comparison. These cell lines were propagated in Dulbecco's modified Eagle's medium or minimum essential medium Eagle containing 10 % (vol/vol) fetal bovine serum, as described (Núñez-Hernández *et al.*, 2014). Bacterial infection assays were performed at small or large scale. For the standard assay (small scale), bacteria were grown for 18 h overnight without shaking in LB medium at 37°C. These bacteria were used to infect the eukaryotic cells as described (Núñez-Hernández *et al.*, 2014). For large-scale experiments, required to monitor protein levels in intracellular bacteria, fibroblast or epithelial cells lines were cultured in 500 cm^2 plates, as described (Núñez-Hernández *et al.*, 2013). Infection conditions and the modified protocol to isolate intact intracellular bacteria were as described (Núñez-Hernández *et al.*, 2013).

Bacterial subcellular fractionation

To assess the location of EcgA, bacteria were grown to stationary phase in LB medium at 37°C, an optimal condition for production of the enzyme. 10^9 bacteria were spun down by centrifugation (4000 × *g*, 5 min, 4°C) and resuspended in 0.5 ml of phosphate buffered saline (PBS), pH 7.4. Isolation of cytosol and inner/outer membrane fractions was as described (Pucciarelli *et al.*, 2002).

EcgA *protein purification*

ecgA coding sequence was cloned in vector pET22(b) + using *E. coli* strain BL21(DE3) as host. Using this cloning strategy, EcgA was detected exclusively in the pellet containing membrane and inclusion bodies, and eventual cell lysis occurred. Similar results were also obtained when using *S.* Typhimurium wild-type strain SV5015 as host. To overcome this, a shorter *ecgA* version lacking the signal peptide (residues 1 to 22) was cloned in *E. coli* BL21(DE3)/pET22. The pET22 plasmid harbours a 6×His tag to fuse with the gene of interest. This shorter EcgA′-6×His version was expressed with a minimal amount of the protein detected in inclusion bodies. EcgA′-6×His protein was purified from the soluble (cytosolic) fraction using TALON metal affinity resin (Clontech).

Enzymatic assays with purified EcgA′-6xHis protein

An amount corresponding to 16 μg of EcgA-6×His was incubated with 24–28 nmoles of the respective muropeptide in 50 mM Tris-HCl pH 8.0, 300 mM NaCl at 37°C for 3 h. The muropeptides tested included: M3 (GlcNAc-MurNAc-L-Ala-D-Glu-dap); M4 (GlcNAc-MurNAc-L-Ala-D-Glu-dap-D-Ala); M5 (GlcNAc-MurNAc-L-Ala-D-Glu-dap-D-Ala-D-Ala); D43 (GlcNAc-MurNAc-L-Ala-D-Glu-dap-D-Ala-dap-D-Glu-L-Ala-NAcMu-GlcNAc); D44 (GlcNAc-MurNAc-L-Ala-D-Glu-dap-D-Ala-dap-(D-Ala)-D-Glu-L-Ala-NAcMu-GlcNAc); and D34D (GlcNAc-MurNAc-L-Ala-D-Glu-dap-dap-(D-Ala)-D-Glu-L-Ala-NAcMu-GlcNAc). The products of the reaction were analysed by reverse phase HPLC (Waters 1525 system) using a Phenomenex Aeris 3.6 μm Peptide XB-C18 (250 × 4.6 mm) column at a flow rate of 1 ml/min and 45°C. Elution was performed by running a 3 min isocratic step in 50 mM sodium phosphate pH 4.35, followed by a 60 min linear gradient to 15% (vol/vol) methanol in 75 mM sodium phosphate pH 4.95. Eluting compounds were detected by monitoring A_{204} (Waters 2489 UV/Vis detector), and quantified using Waters Breeze 2 software (Waters Corporation, Milford, MA, USA). EcgA activity was also tested against macromolecular peptidoglycan, which was purified as described (Quintela *et al.*, 1997). Peptidoglycan obtained from 5 ×10^{10} bacteria was incubated with 100 μg of EcgA′-6×His at 37°C overnight in buffer 50 mM Tris pH 8.0, 300 mM NaCl. Peptidoglycan was digested with muramidase and processed for HPLC analysis as described (Quintela *et al.*, 1997).

Antibodies and western analysis

Primary antibodies used for Western blotting included mouse monoclonal anti-FLAG epitope (clone M2, Sigma) and rabbit polyclonal anti-IgaA (Dominguez-Bernal *et al.*, 2004). Goat polyclonal anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) were used as secondary antibodies at a 1:5000 dilution. SDS-PAGE and western blotting were as described (Núñez-Hernández *et al.*, 2014).

BALB/c mice infection experiments

Oral and intraperitoneal challenge of 7- to 8-week-old female BALB/c mice was performed as described (Dominguez-Bernal *et al.*, 2004). These experiments involved the use of bacterial mixtures in which the ratio of wild-type and Δ*ecgA*::Km strains was determined in both the input and output. Groups of six and four animals were infected by the oral and intraperitoneal route respectively. The doses used in the input mixture were 5×10^8 cfu in 25 μl PBS-0.1% lactose in oral challenges and 10^4 cfu in 200 μl of PBS for i.p. challenges. Competitive index was calculated at 5 days (oral challenge) or 48 h (i.p. challenge) as described (Dominguez-Bernal *et al.*, 2004).

Statistical analysis

Data were analysed by *t*-test using Prism version 5.0 (GraphPad Software). Differences in the values with $P < 0.05$ were considered significant.

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The authors declare no conflict of interest

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Supporting information

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