

The stress-responsive Hsp90 chaperone is required for the production of the genotoxin colibactin and the siderophore yersiniabactin by *Escherichia coli*

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ABSTRACT (195 words)

The genotoxin colibactin synthesized by *Escherichia coli* is a secondary metabolite belonging to the chemical family of hybrid polyketide/non-ribosomal peptide compounds. It is produced by a complex biosynthetic assembly line encoded by the *pks* pathogenicity island. The presence of this large cluster of genes in the *E. coli* genome is invariably associated with the High-Pathogenicity Island, encoding the siderophore yersiniabactin that belongs to the same chemical family as colibactin. The *E. coli* heat shock protein HtpG (Hsp90_{Ec}) is the bacterial homolog of the eukaryotic molecular chaperone Hsp90 involved in the protection of cellular proteins against a variety of environmental stresses. In contrast to the eukaryotic Hsp90, the functions and client proteins of Hsp90_{Ec} are poorly known. Here, we demonstrated that production of colibactin and yersiniabactin is abolished in the absence of Hsp90_{Ec}. We further characterized an interplay between the Hsp90_{Ec} molecular chaperone and the ClpQ protease involved in colibactin and yersiniabactin synthesis. Finally, we demonstrated that Hsp90_{Ec} is required for the full in vivo virulence of extraintestinal pathogenic *E. coli*. This is the first report highlighting the role of heat shock protein Hsp90_{Ec} in the production of two secondary metabolites involved in *E. coli* virulence.

INTRODUCTION (3,335 words)

Escherichia coli is both a commensal inhabitant of the human gastrointestinal tract and a pathogen associated with a wide range of infections. Certain pathogenic *E. coli* strains, *i.e.* Extraintestinal Pathogenic *E. coli* (ExPEC), display an enhanced capacity to cause infection outside the intestinal tract. These strains harbor numerous virulence factors encoded by mobile genetic elements, such as plasmids, transposons, phages or pathogenicity islands [1].

We previously revealed the presence in the *E. coli* genome of a 54 kb gene cluster, the *pks* genomic island [2]. This highly conserved pathogenicity island is predominately found in *E. coli* strains of the phylogenetic group B2 and in some other pathogenic species of *Enterobacteriaceae*, such as *Citrobacter koseri*, *Klebsiella pneumoniae* or *Enterobacter aerogenes* [2,3]. The *pks* island carries genes *clbA* to *clbS*, and encodes modular non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs) and accessory enzymes. This complex biosynthetic machinery synthesizes a microbial secondary metabolite, the genotoxin colibactin, which is a hybrid polyketide/non-ribosomal peptide (PK-NRP) compound. Colibactin was demonstrated to generate DNA double strand breaks in eukaryotic cells both in vitro and in vivo [2–5]. This DNA damage leads to mutations, chromosomal instability and premature senescence that could ultimately drive tumorigenesis [4,6,7]. *E. coli* strains also synthesize other PK-NRP secondary metabolites, including the siderophores enterobactin, salmochelins and yersiniabactin. Siderophores are low molecular weight compounds that facilitate iron uptake by bacteria, and are virulence factors [8]. We recently described a crosstalk between the biosynthesis of the PK-NRP secondary metabolites colibactin and siderophores [9]. Our recent studies have also demonstrated the importance of the interplay between colibactin and siderophores in the virulence of ExPEC in rodent models of sepsis and neonatal meningitis [5,9,10].

Entry of a pathogen into a warm-blooded host is usually accompanied by a temperature upshift. In bacteria, such change triggers a global stress response, named the heat shock response, which is orchestrated by heat shock proteins (HSPs) [11]. HSPs include highly conserved molecular

chaperones, proteases, transcription factors and key metabolic enzymes that facilitate adaptation and survival in response to exogenous stressors [12]. Heat shock genes are also induced at later stages of infection, reflecting their role in the protection of the bacterium against a variety of other stresses and host defense mechanisms, such as oxidative stress, low pH, defensins or bactericidal serum activity [13]. These processes are essential for the survival of the pathogen within the host.

Molecular chaperones are ubiquitous and highly conserved proteins that maintain intracellular protein homeostasis. They assist folding, translocation, quality control, targeting to proteases, assembly and disassembly of protein complexes [14,15]. One of these chaperones, the High temperature protein G (HtpG), is the bacterial homolog of the eukaryotic Heat shock protein 90 (Hsp90). The Hsp90 machinery is involved in diverse cellular processes including protein folding or repairing and signal transduction [16,17], and has been demonstrated as a key therapeutic target in cancers or neurodegenerative diseases [18,19]. However, in contrast to the essential nature of eukaryotic Hsp90, deletion of the *htpG* gene is not lethal to bacterial cells, but results in impaired growth at high temperatures [20,21]. Furthermore, cellular functions and client proteins of the Hsp90 bacterial homolog remain enigmatic, despite its high conservation among bacteria and high abundance in the cell [22,23]. In this work, we demonstrate that the *E. coli* molecular chaperone HtpG, thereafter called Hsp90_{Ec}, is required for the synthesis of both colibactin and yersiniabactin, and is involved in the extraintestinal virulence of *E. coli*. These results confirm the role of the stress response during infection, and indicate that bacterial Hsp90 should be considered a potential therapeutic target for antimicrobial treatments.

MATERIAL AND METHODS

***N*-myristoyl-D-asparagine (colibactin prodrug motif) quantification by liquid chromatography/mass spectrometry**

Strains were grown in DMEM medium at 37°C for 18 h (see Supplementary Methods for details). Supernatants of cultures were obtained following centrifugation of bacterial cells at 3,200 ×

g for 15 min and were filtered on 0.2 μm membranes. Aliquots of 1 mL of supernatant were prepared. Each strain was cultured in triplicate (deriving from three independent clones) and each supernatant was analyzed by LC-MS/MS.

Quantification experiments were conducted with ultra-performance liquid chromatography-high resolution/heated electrospray ionization mass spectrometry (UPLC-HR/HESI-MS). The data were recorded on a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled to a Dionex Ultimate 3000 UPLC. The following solvent gradient (A = H_2O + 0.1% formic acid, B = acetonitrile + 0.1% formic acid with B at 30% from 0-1 min, 30-95% from 1-6 min and 95% from 6-7 min at a flowrate of 0.5 mL/min) was used on a Phenomenex Kinetex 5 μm EVO C18 (50 \times 2.1 mm) column at 30°C. The MS was operated in positive ionization mode at a scan range of 200-500 m/z and a resolution of 35,000. The spray voltage was set to 3.5 kV, the S-lens to 35, the auxiliary gas heater temperature to 438°C and the capillary temperature to 270°C. Absolute quantification was achieved by using a Schotten-Baumann reaction-derived *N*-myristoyl-L-asparagine (isomer of the *N*-myristoyl-D-asparagine colibactin cleavage product) as a standard. The standard curve was recorded with methanol-diluted concentrations of 10 ng/mL, 100 ng/mL, 500 ng/mL, 1 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$ from a 1 mg/mL methanol stock solution. Data were obtained from undiluted cell free sample supernatants and analyzed for *N*-myristoyl-D-asparagine and concentrations were calculated using Thermo Xcalibur 2.2 Quan Browser.



Siderophores quantification by liquid chromatography/mass spectrometry

Culture supernatants were obtained following centrifugation of bacterial cells at 3,200 \times g for 15 min and were filtered on 0.2 μm membranes (see Supplementary Methods). Aliquots of 1 mL of supernatant were prepared, and 0.12 ng/mL of 5,6,7,8-tetradeutero-3,4-dihydroxy-2-heptylquinoline was added as an internal control. Each strain was cultured in triplicate and each culture supernatant was analyzed by LC-MS/MS.

Multiple-reaction-monitoring (MRM) analyses were performed using a Waters 2795 Alliance HT high-performance liquid chromatography (HPLC) system coupled to a Micromass Quattro Premier XE mass spectrometer (Micromass MS Technologies). Samples were injected onto a Phenomenex Kinetex 2,6u C8 100A by 150-mm column at a flow rate of 400 μ L/min and with a linear gradient of water-acetonitrile with 1% acetic acid. The transition for yersiniabactin was m/z 482 > 295. The specific transitions from pseudomolecular to daughter ions of salmochelins, enterobactin, and aerobactin are described elsewhere [24]. These transitions were used for relative quantification.

Mouse sepsis model

The procedure has been described in detail previously [5]. Briefly, nine-week-old female C57BL/6J mice (JANVIER) were injected into the footpad with 10^8 SP15 wild type and $\Delta htpG$ mutant strains (see Supplementary Methods). 20 hours after injection, mice were treated with 100 μ L of 1 mg/mL gentamicin injected intraperitoneally, together with ringer solution injected subcutaneously ($2 \times 500 \mu$ L) for rehydration.

Rat neonatal meningitis model

The procedure has been described in detail previously [25]. Briefly, all members of a litter ($n = 12$) of two-day-old (P2) Wistar rat pups (Harlan, United Kingdom) were fed 20 μ L of mid-logarithmic-phase *E. coli* bacteria (6×10^6 CFU) from an Eppendorf micropipette to induce gastrointestinal colonization (see Supplementary Methods). Disease progression was determined by daily evaluation of all rat pups for symptoms of systemic infection and scored on a scale of rising severity from 0 to 3. Pups scoring 3 were culled, and systemic infection was confirmed by quantifying *E. coli* K1 in blood samples on MacConkey agar and the expression of the K1 capsule confirmed by testing susceptibility of colonies to bacteriophage K1E.

Ethic statement

Animal experiments were carried out in accordance with the European directive for the protection of animals used for scientific purposes. Mouse experiments were approved by the local ethic committee on animal experiment "Comité d'éthique pour la protection de l'animal de laboratoire Midi-Pyrénées (C2EA-22)" and were conducted under the referenced protocol MP/03/63/07/12. Rat experiments were approved by the Ethical Committee of the UCL School of Pharmacy and the United Kingdom Home Office (HO) and were conducted under the HO Project License PPL 70/7773.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 6.0c. The mean and the standard error of the mean (SEM) are shown in figures. *p*-values were calculated using unpaired t-test or one-way ANOVA test followed by a Bonferroni post-test. For in vivo experiments, survival curves were analyzed using log-rank test. A *p*-value of less than 0.05 was considered statistically significant and is denoted by *. *p* < 0.01 is denoted by ** and *p* < 0.001 by ***. Non-significant result is indicated *ns*.

RESULTS

The molecular chaperone Hsp90_{Ec} is required for the genotoxicity of *pks*⁺ *E. coli*

The *pks* island was initially identified as the genomic determinant for the synthesis of colibactin through the screening of a transposon mutant library [2]. Interestingly, screening of this library had revealed that several transposon mutants had insertions in the *htpG* gene, suggesting that *htpG* was potentially involved in colibactin biosynthesis (unpublished data). To investigate the impact of Hsp90_{Ec} in colibactin production, a deletion of the *htpG* gene was constructed (see Supplementary Methods) in commensal and pathogenic *pks*⁺ *E. coli* strains M1/5 and SP15, respectively (Supplementary Table 1). The resulting Δ *htpG* mutant strains were subsequently complemented

with the plasmid p-*htpG* harboring the *htpG* gene under the control of an IPTG-inducible promoter (Supplementary Table 1). The production of colibactin was measured in the mutant and complemented strains through bacteria-host cells interactions and subsequent quantification of megalocytosis (Figure 1A) and histone H2AX phosphorylation (Figure 1B), which correlate with DNA double strand breaks resulting from the genotoxic effect of colibactin [2,4].

The megalocytosis assay [2] and the quantification of H2AX phosphorylation using an In-Cell Western assay [26] revealed that the inactivation of the *htpG* gene in both the M1/5 and SP15 strains abrogated the genotoxic effect induced by the colibactin (Figure 1). Transformation of the Δ *htpG* mutants with p-*htpG* carrying the functional wild type *htpG* gene resulted in a complete restoration of the genotoxicity (Figure 1). Altogether, these data demonstrated that Hsp90_{Ec} is required for colibactin mediated genotoxicity.

Hsp90_{Ec} is directly involved in colibactin biosynthesis

The mature colibactin genotoxin has not been fully characterized yet. However, a biosynthetic by-product derived from the colibactin assembly line, *i.e.* the N-myristoyl-D-asparagine moiety, has been recently characterized [27,28]. This moiety, generated by the ClbN enzyme, is a prodrug motif cleaved by the ClbP peptidase in the late activation step. We developed a LC-MS assay to quantitatively measure the amount of N-myristoyl-D-asparagine as a means to indirectly quantify the production of the genotoxin by wild type and Δ *htpG* mutant in *E. coli* M1/5 (Figure 2). This revealed that the amount of colibactin prodrug motif was not detectable in the Δ *htpG* mutant compared to the wild type strain (Figure 2). These results indicated that Hsp90_{Ec} was directly or indirectly required for the synthesis of the biosynthetic intermediate N-myristoyl-D-asparagine, and therefore for colibactin biosynthesis.

Hsp90_{Ec} does not regulate transcription of the *pks* island genes

To investigate whether Hsp90_{Ec} was involved in the regulation of colibactin production via

transcriptional regulation of colibactin biosynthesis genes, we quantified the transcription of *clb* genes encoded on the *pks* island, in relation to *htpG* expression (Supplementary Methods, Figure 3). M1/5 wild type strain and its Δ *htpG* derivative were grown at 37°C and then shifted to 45°C to provoke a heat shock stress, known to induce Hsp90_{Ec} production [29]. Total RNA was isolated and was used for a transcriptional analysis of the *htpG* gene and genes *clbA*, *clbC*, *clbJ*, *clbP* and *clpQ*, selected to monitor the expression of the different transcriptional units identified in the *pks* island [30]. This revealed that a transient heat shock resulted in an increased transcription of *htpG* (Figure 3), as previously shown [29]. Nonetheless, the transcription of the *clb* genes was not altered in response to a heat shock stress, and was unchanged whether *htpG* was functional or inactivated (Figure 3). These data suggested that Hsp90_{Ec} was not involved in the transcription of the *pks* island genes.

Hsp90_{Ec} is involved in yersiniabactin production

E. coli possesses biosynthetic pathways that yield compounds belonging to the same chemical family as colibactin, *i.e.* siderophores, which mediate bacterial uptake of iron and other metals [8]. *E. coli* strains are known to synthesize up to four different types of siderophores: aerobactin, enterobactin, salmochelins and yersiniabactin, which are PK-NRP compounds except for aerobactin. To determine whether Hsp90_{Ec} is also involved in siderophore production, we quantified by LC-MS the amount of each siderophore [31] synthesized by wild type or Δ *htpG* mutant *E. coli* M1/5 and SP15 strains (Figure 4). This revealed that the synthesis of yersiniabactin was decreased in both M1/5 Δ *htpG* and SP15 Δ *htpG* mutant strains compared to the wild type strains. The amount of the other siderophores remained unchanged whether *htpG* was disrupted or not (Figure 4). These data indicated that Hsp90_{Ec} also contributed to the biosynthesis or accumulation of yersiniabactin.

Interplay between Hsp90_{Ec} and the ClpQ protease modulates colibactin and yersiniabactin biosynthesis

Specific interplays between Hsp90_{Ec} and the chaperone/protease network were previously reported [32,33]. In order to assess whether the absence of Hsp90_{Ec} would abolish competition with proteases for colibactin-involved client binding, we tested the effect of the disruption of the three major *E. coli* cytosolic stress proteases Lon, ClpP and ClpQ (also called HslV) on the $\Delta htpG$ mutant phenotype. The *lon*, *clpP* or *clpQ* (or *hslV*) genes were individually inactivated in the M1/5 $\Delta htpG$ mutant strain (see Supplementary Methods). The resulting double mutants (Supplementary Table 1) were analyzed for the production of colibactin by quantification of the megalocytosis phenotype (Figure 5A), quantification of colibactin prodrug motif synthesis (Figure 5B), and for the production of yersiniabactin (Figure 5C).

The megalocytosis assay revealed that inactivation of the *lon* or *clpP* genes did not restore the genotoxic effect of colibactin in the $\Delta htpG$ mutant (Figure 5A). On the contrary, infection of HeLa cells with the $\Delta htpG \Delta clpQ$ double mutant resulted in a restored megalocytosis phenotype, indicating the production of colibactin (Figure 5A). Transformation of this double mutant with a plasmid carrying the functional *clpYQ* operon (p-*clpYQ*, Supplementary Table 1) resulted in a decreased colibactin activity (Figure 5A). We then analyzed a $\Delta clpQ$ mutant in *E. coli* strain M1/5 (Supplementary Table 1) for colibactin production in the megalocytosis assay (Figure 5A). Inactivation of the *clpQ* gene resulted in a colibactin production level similar to the wild type strain (Figure 5A). When the $\Delta clpQ$ mutant was transformed with an inducible and high copy number plasmid carrying the functional *clpYQ* operon (p-*clpYQ*, Supplementary Table 1), the resulting complemented derivative lost the ability to induce megalocytosis (Figure 5A). Moreover, introduction of the same plasmid in wild type strain also resulted in the loss of the megalocytosis phenotype.

We quantified by LC-MS the amount of N-myristoyl-D-asparagine (Figure 5B) and the production of yersiniabactin (Figure 5C) synthesized by $\Delta htpG \Delta clpQ$ and $\Delta clpQ$ mutants and

complemented derivatives of *E. coli* M1/5. Patterns similar to those obtained in the megalocytosis experiments (Figure 5A) were observed.

Altogether, these results indicated that disruption of the ClpQ protease allowed restoration of colibactin and yersiniabactin production in the absence of Hsp90_{Ec}. These data suggested that both the Hsp90_{Ec} molecular chaperone and the ClpQ protease might share specific substrate(s) of the colibactin and the yersiniabactin biosynthesis pathways, and that Hsp90_{Ec} could protect the substrate(s) from ClpQ-mediated degradation.

Hsp90_{Ec} is required for full virulence of ExPEC in a meningitis infection model

Colibactin and yersiniabactin are *bona fide* virulence factors [5,8–10]. To address the biological relevance of the chaperone protein Hsp90_{Ec} on *E. coli* virulence in vivo, we analyzed the effects of the *htpG* gene disruption during systemic infection in animals, using a mouse model of sepsis and a rat model of neonatal meningitis (Figure 6).

SP15 wild type and Δ *htpG* mutant *E. coli* strains were injected into mice footpads to induce sepsis, as previously described [5]. Infected mice were then treated with antibiotics and hydration 20 hours post-infection. Monitoring of animal survival revealed that the mortality of mice inoculated with wild type SP15 appeared higher than that in the SP15 Δ *htpG* group (Figure 6A); however, the log-rank test was not statistically significant.

The second infection model tested the ability of wild type and Δ *htpG* mutant *E. coli* A192PP strains to induce systemic infection in neonatal rats following oral feeding of live A192PP bacteria [34]. The wild type *E. coli* A192PP strain produced lethal infection in all colonized pups. On the contrary, although a proportion of pups colonized with the A192PP Δ *htpG* mutant did not survive, the overall lethal effect of this mutation was significantly attenuated ($p = 0.0032$), with an increased survival of about 20% (Figure 6B). This demonstrated that Hsp90_{Ec} chaperone protein was required to maintain full virulence of ExPEC during systemic infection.

DISCUSSION

In this study, we demonstrated that Hsp90_{Ec} is mandatory for the production of two virulence factors produced by *E. coli*, the genotoxin colibactin and the siderophore yersiniabactin. Thus, our work provides new insights into the role of the Hsp90_{Ec} molecular chaperone, together with the recent studies that highlighted Hsp90_{Ec} functions in *E. coli* [21,32,35,36]. Other bacterial Hsp90 were previously shown to be required for the biosynthesis of PK-NRP compounds, such as albicidin, an antibiotic and phytotoxin produced by *Xanthomonas albilineans* [37], or arthrobactin, a biosurfactant produced by *Pseudomonas* strains [38]. Based on these studies, we could hypothesize that the molecular chaperone Hsp90_{Ec} either facilitates the folding or prevents a rapid degradation of a colibactin-synthesis enzyme(s), as proposed for the biosynthesis of arthrobactin in *Pseudomonas* sp. [38]. Moreover, disruption of the *clpQ* gene allowed a restoration of colibactin and yersiniabactin biosynthesis in the absence of Hsp90_{Ec}, which suggests that the chaperone and the protease could share common client protein(s) in the biosynthetic pathways. The fact that overexpression of the *clpYQ* operon induced a decrease of colibactin-mediated genotoxicity is in agreement with the substrate overlap, and suggests that Hsp90_{Ec} could protect a substrate required for colibactin production from degradation by the ClpQ protease. The interplay between Hsp90_{Ec} and ClpQ might be an efficient way to posttranslationally control colibactin synthesis. Unfortunately, we have not yet identified a specific client protein involved in colibactin and yersiniabactin biosynthesis directly interacting with Hsp90_{Ec}. So far, only two characterized client proteins for Hsp90_{Ec} have been reported in *E. coli*, the ribosomal protein L2 [39] and the DNA-replication initiator DnaA [40]. The incapacity to identify a specific partner in these pathways could also be explained by a more general function of the chaperone. We indeed hypothesize that Hsp90_{Ec} participates in the assembly or stabilization of the biosynthetic machinery complex, as it has been proposed for the biosynthesis of albicidin in *Xanthomonas albilineans*, where no HtpG-specific client protein has been identified [37].

Colibactin and yersiniabactin have been characterized as virulence factors in vivo [5,8–10].

Here, we demonstrated that Hsp90_{Ec} is required for full virulence of ExPEC in a model of neonatal meningitis, thus linking a major stress-induced molecular chaperone involved in protein homeostasis to a successful infection. Our study highlights for the first time the role of Hsp90_{Ec} in *E. coli* virulence. In other bacterial species, HtpG has been demonstrated to be involved in the virulence of *Edwardsiella tarda* [41], *Francisella tularensis* [42], *Leptospira interrogans* [43] and *Salmonella typhimurium* [44]. Here, we showed that Hsp90_{Ec} is required for successful infection in a rat model of neonatal meningitis, which support the fact that bacterial stress adaptation through the global heat shock response could be essential for a successful infectious process.

The involvement of HtpG in the virulence of various bacterial species led us to propose bacterial Hsp90 as a potential antimicrobial therapeutic target, as described for eukaryotic Hsp90 in the treatment of various human cancers [18], but also protozoan [45] and fungal [46] infections. Geldanamycin and radicicol are two natural products that have both been shown to inhibit the ATPase activity and function of the eukaryotic Hsp90 chaperone [18]. Both compounds have also been demonstrated to inhibit bacterial Hsp90 [47,48]. The use of these two inhibitors in our animal models would provide us with invaluable information. The major challenge of this promising strategy would be to develop Hsp90 inhibitors specific for the prokaryotic isoform of the molecular chaperone to avoid side effects of antimicrobial treatment by eukaryotic Hsp90 inhibition. Additional knowledge about Hsp90_{Ec} will be required to reach that objective.

NOTES

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Potential conflict of interest

The authors have no conflict of interest to declare.

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FIGURE LEGENDS

Figure 1. The molecular chaperone Hsp90_{Ec} (HtpG) is required for *Escherichia coli* genotoxicity.

The production of colibactin by *E. coli* strains M1/5 and SP15 derivatives was determined by quantification of megalocytosis (A) and of H2AX phosphorylation (B). *E. coli* wild type strain, $\Delta htpG$ mutants and complemented derivatives were cocultivated with HeLa cells for 4 h, then washed as previously described [2]. At the end of infection, bacterial growth was similar for all strains. A, After infection, the cells were incubated for 72 h with appropriate antibiotics before protein staining with methylene blue. The staining was quantified by acid-extraction of methylene blue and measurement of absorbance at an optical density of 660 nm (OD_{660 nm}). Multiplicity of infection: MOI = 200. Statistical analysis: one-way Anova. ***: $p < 0.001$, ns: not significant. B, After infection, the cells were incubated 4 h in DMEM medium supplemented with antibiotics before fixation, then permeabilized and labeled for DNA (pseudo-colored red) and phosphorylated H2AX histone (γ -H2AX, pseudo-colored green) using an In-Cell Western method [26]. MOI = 50 to 12.

Figure 2. Hsp90_{Ec} required for the synthesis of colibactin prodrug motif. The colibactin prodrug motif *N*-myristoyl-D-asparagine produced by *E. coli* strain M1/5 wild type and $\Delta htpG$ mutant was quantified by LC-MS. Bacteria were cultivated at 37°C for 18 h in DMEM medium, and *N*-myristoyl-D-asparagine was quantified in culture supernatants by LC-MS using *N*-myristoyl-L-asparagine (isomer of the *N*-myristoyl-D-asparagine colibactin prodrug motif) as a standard. The results were normalized to the bacterial biomass and are presented as quantity of *N*-myristoyl-D-asparagine (ng/mL). Data represented in the graph were obtained from three biological replicates. Similar results were observed for two independent experiments. Statistical analysis: unpaired t-test. ***: $p < 0.001$, **: $p < 0.01$, ns: not significant.

Figure 3. Hsp90_{Ec} (HtpG) does not regulate the transcription of genes located on the *pks* island.

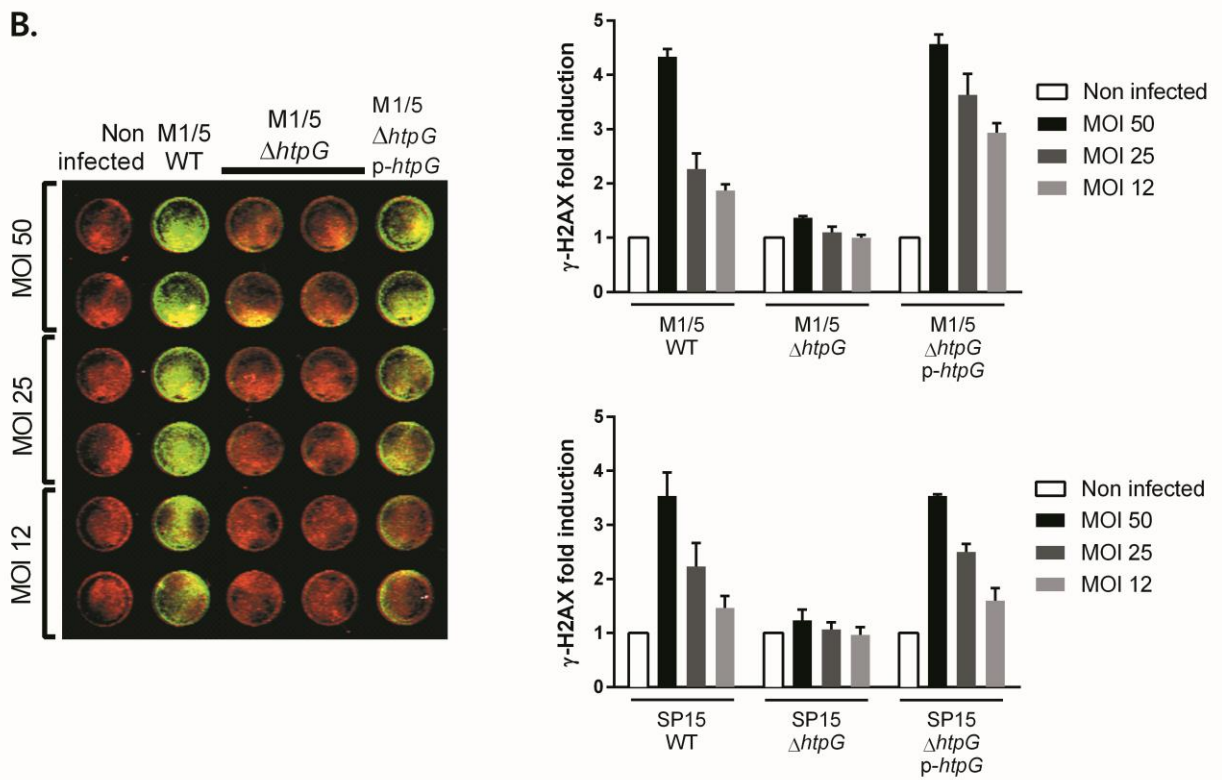
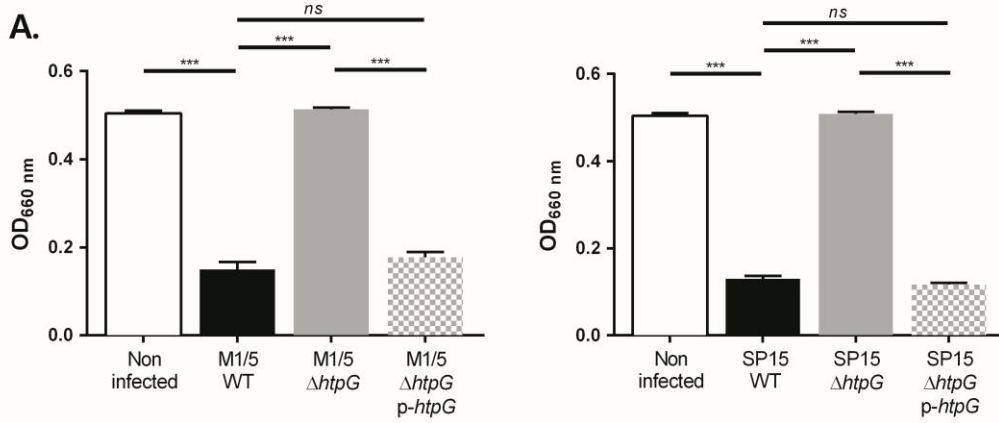
Gene expression level of *htpG* and five *clb* genes of the *pks* island during a heat shock was measured by quantitative RT-PCR. *E. coli* strain M1/5 wild type or Δ *htpG* mutant were cultivated at 37°C for 3 hours. A fraction of the cultures was transferred at 45°C during 30 minutes to induce a heat shock. After total RNA extraction, transcription level of *htpG*, *clbA*, *clbC*, *clbJ*, *clbP* and *clbQ* genes was determined by qRT-PCR. Results were normalized to *hcaT* reference gene expression and are presented as increases (*n*-fold) in expression level compared to that of M1/5 wild type strain cultivated at 37°C. Statistical analysis: one-way Anova. ***: $p < 0.001$.

Figure 4. Hsp90_{Ec} is also involved in yersiniabactin production. Siderophore production by *E. coli* strains M1/5, SP15 and derivatives was quantified by LC-MS. *E. coli* strains M1/5 and SP15 wild type and Δ *htpG* mutant were cultivated at 37°C for 18 h in DMEM medium. Siderophore production was quantified by LC-MS, as described previously [31]. The results were normalized to the bacterial biomass and are presented as peak surfaces. Data represented in the graph were obtained from three biological replicates. Similar results were observed for two independent experiments. Statistical analysis: unpaired t-test. ***: $p < 0.001$.

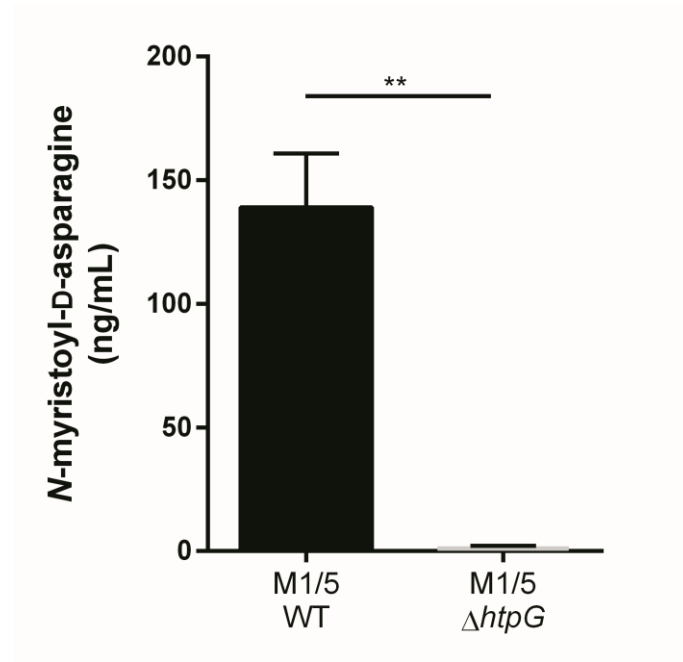
Figure 5. The protease ClpQ is involved in colibactin and yersiniabactin production in combination with Hsp90_{Ec}. *A*, Colibactin-mediated genotoxicity was determined by infection of HeLa cells and quantification of megalocytosis for *E. coli* strain M1/5 and derivatives, as described in Figure 1A. Multiplicity of infection: MOI = 200. Statistical analysis: one-way Anova. ***: $p < 0.001$, *ns*: not significant. *B*, Colibactin prodrug motif *N*-myristoyl-D-asparagine production by *E. coli* strain M1/5 and derivatives was quantified by LC-MS, as described in Figure 2. The results were normalized to the bacterial biomass and are presented as quantity of *N*-myristoyl-D-asparagine (ng/mL). Statistical analysis: one-way Anova. **: $p < 0.01$, *ns*: not significant. *C*, Yersiniabactin production by *E. coli* strains M1/5 and derivatives was quantified by LC-MS, as described in Figure 4. The results were

normalized to bacterial biomass and are presented as peak surfaces. Statistical analysis: one-way Anova. ***: $p < 0.001$, **: $p < 0.01$, *ns*: not significant.

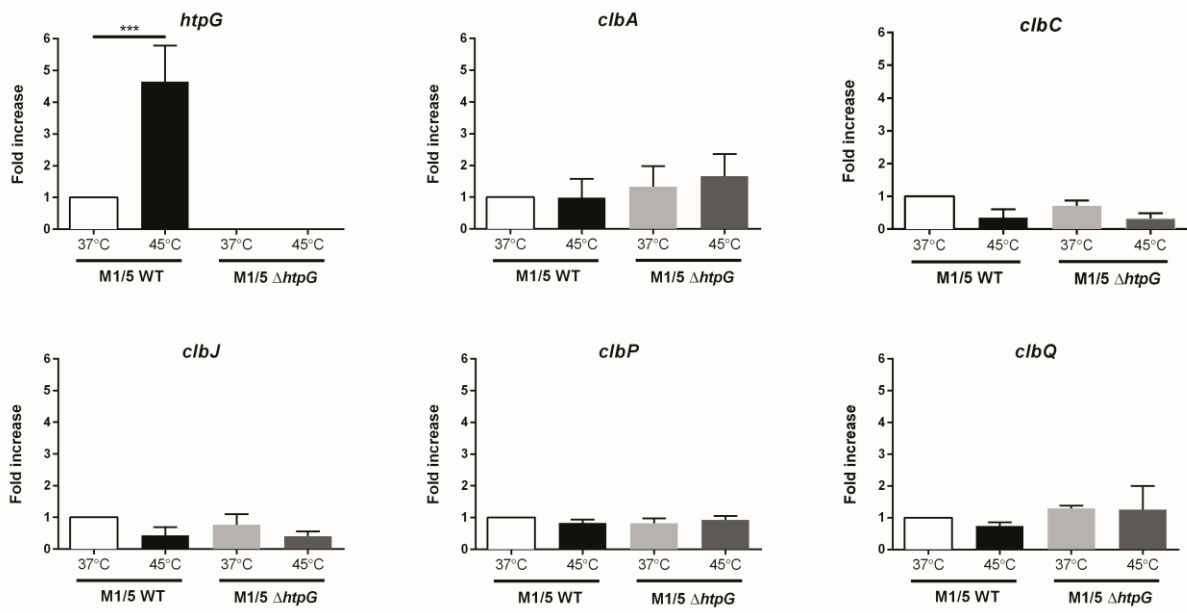
Figure 6. Effect of *htpG* inactivation in in vivo models of systemic infection. *A*, Virulence of *E. coli* strain SP15 wild type and Δ *htpG* mutant was evaluated in a murine model of sepsis with antibiotic rescue [5]. Mice received footpad injection with PBS or 10^8 CFU of *E. coli* SP15 wild type strain or Δ *htpG* mutant. Mice were then treated with gentamicin (100 μ g per mouse) 20 hours post-injection. Percentage of mice survival was monitored for the different groups ($n = 15$ /group). The data shown in the graph are pooled data obtained from two independent experiments. Statistical analysis: log-rank test. *ns*: not significant ($p = 0.117$). *B*, Virulence of *E. coli* strain A192PP and Δ *htpG* mutant was evaluated in a rat model of neonatal meningitis [10]. Two-day-old rats received an orally fed $2-6 \times 10^6$ CFU of *E. coli* strain A192PP wild type or Δ *htpG* mutant. Percentage of rat survival was monitored for the different groups ($n = 12$ /group). The data shown in the graph are pooled data obtained from three independent experiments. Statistical analysis: log-rank test. **: $p < 0.005$ ($p = 0.0031$).



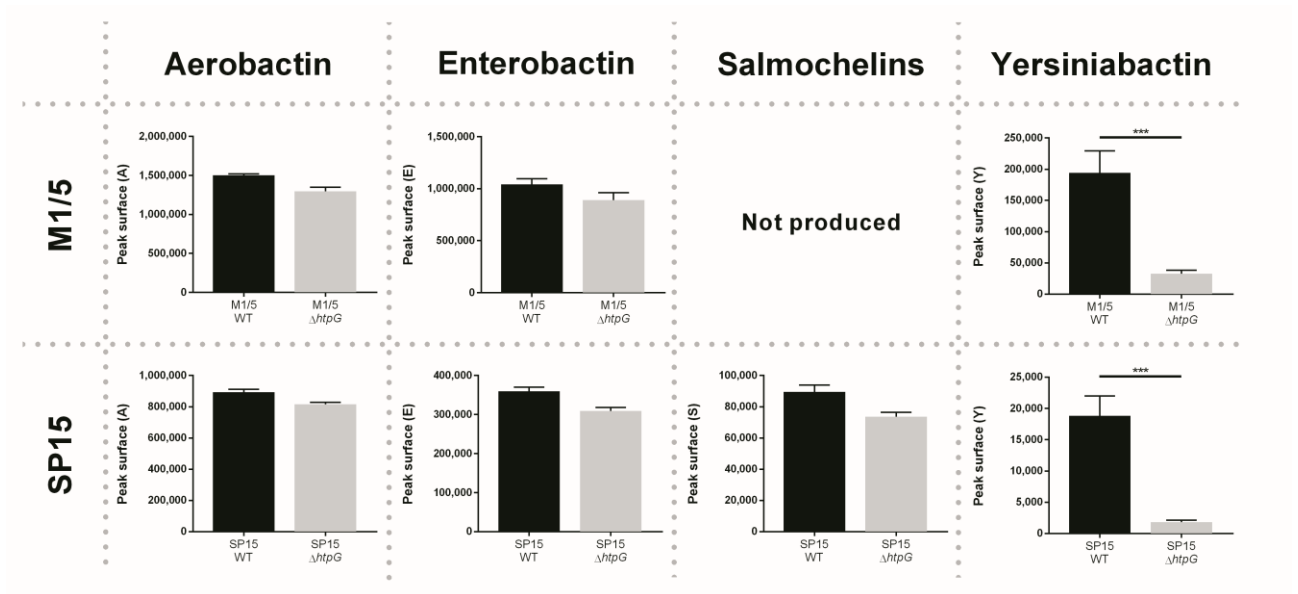
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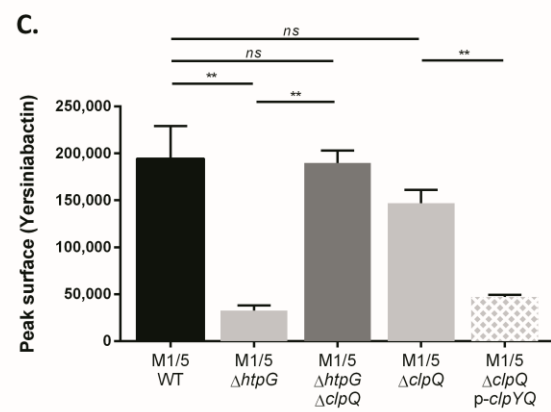
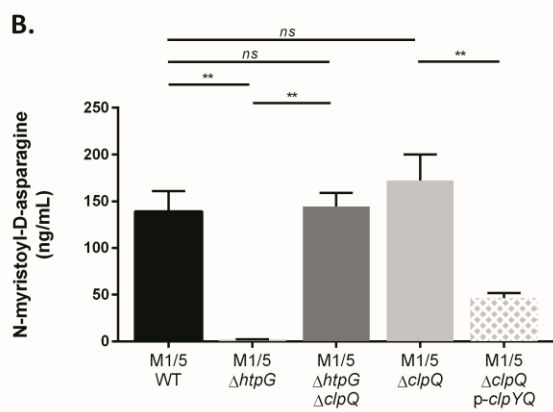
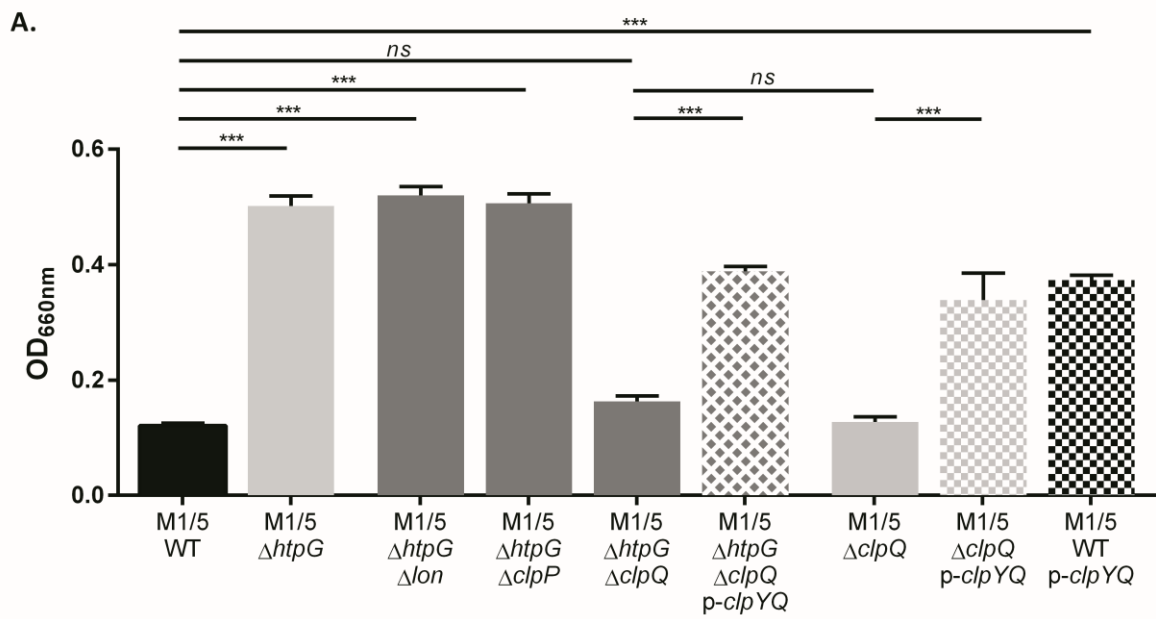
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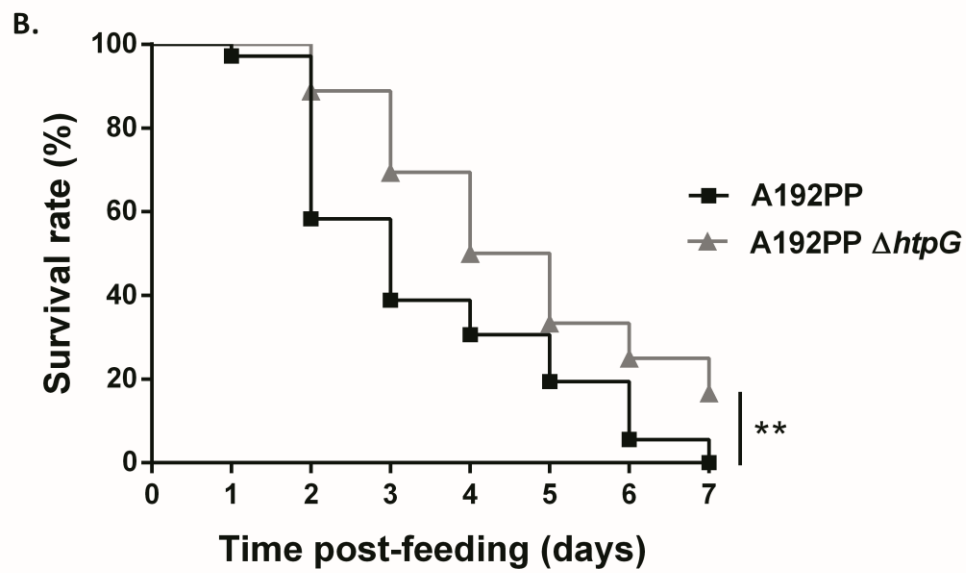
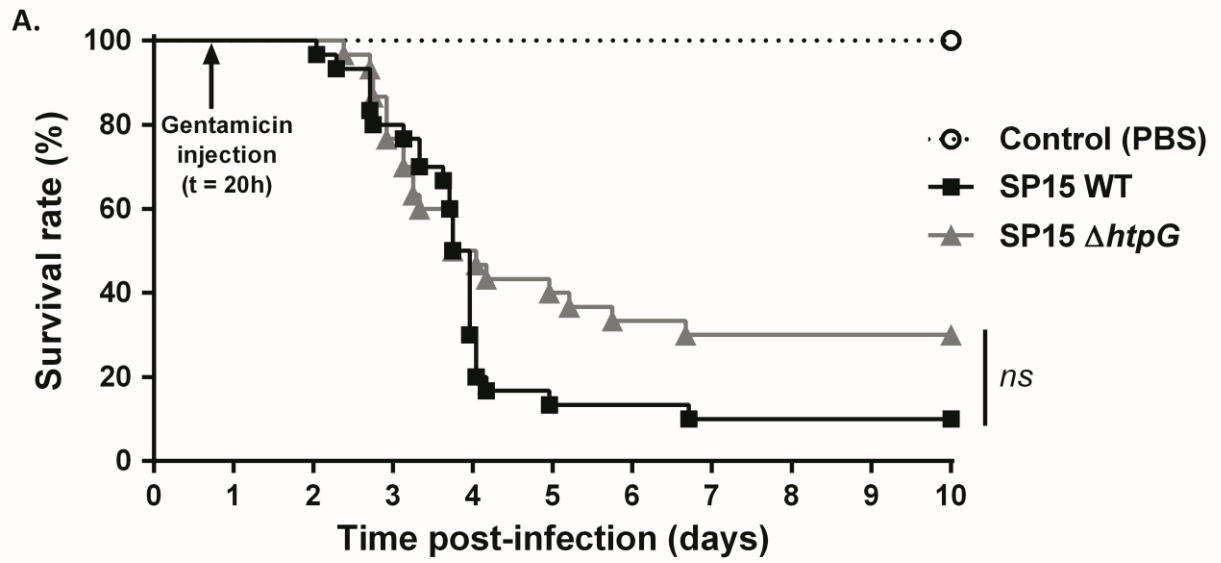
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