Interplay between Siderophores and Colibactin Genotoxin Biosynthetic Pathways in *Escherichia coli*

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Abstract

In *Escherichia coli*, the biosynthetic pathways of several small iron-scavenging molecules known as siderophores (enterobactin, salmochelins and yersiniabactin) and of a genotoxin (colibactin) are known to require a 4′-phosphopantetheinyl transferase (PPTase). Only two PPTases have been clearly identified: EntD and ClbA. The gene coding for EntD is part of the core genome of *E. coli*, whereas ClbA is encoded on the pks pathogenicity island which codes for colibactin. Interestingly, the pks island is physically associated with the high pathogenicity island (HPI) in a subset of highly virulent *E. coli* strains. The HPI carries the gene cluster required for yersiniabactin synthesis except for a gene coding its cognate PPTase. Here we investigated a potential interplay between the synthesis pathways leading to the production of siderophores and colibactin, through a functional interconnection between EntD and ClbA. We demonstrated that ClbA could contribute to siderophores synthesis. Inactivation of both entD and clbA abolished the virulence of extra-intestinal pathogenic *E. coli* (ExPEC) in a mouse sepsis model, and the presence of either functional EntD or ClbA was required for the survival of ExPEC *in vivo*. This is the first report demonstrating a connection between multiple phosphopantetheinyln-requiring pathways leading to the biosynthesis of functionally distinct secondary metabolites in a given microorganism. Therefore, we hypothesize that the strict association of the pks island with HPI has been selected in highly virulent *E. coli* because ClbA is a promiscuous PPTase that can contribute to the synthesis of both the genotoxin and siderophores. The data highlight the complex regulatory interaction of various virulence features with different functions. The identification of key points of these networks is not only essential to the understanding of ExPEC virulence but also an attractive and promising target for the development of anti-virulence therapy strategies.

Introduction

*Escherichia coli* is a normal resident of the lower-gut of humans and animals. Although usually a commensal, *E. coli* can be also a pathogen, associated with diarrheal disease and extra-intestinal infections [1,2]. The majority of *E. coli* strains can be assigned to one of five main phylogenetic groups: A, B1, B2, D and E [3]. Strains of the distinct phylogenetic groups differ in their phenotypic and genotypic characteristics [4–6]. Extra-intestinal pathogenic *E. coli* (ExPEC), which display enhanced ability to cause infection outside the intestinal tract, carry specific genetic determinants or virulence factors that are clustered on different pathogenicity islands [7]. These virulence factors associated with extra-intestinal infections are nonrandomly distributed, and strains of the *E. coli* phylogenetic group B2 harbor the greatest frequency and diversity of virulence traits [8,9].

As iron bioavailability is limited in the host, ExPEC are known to synthesize up to four types of siderophores involved in iron uptake: enterobactin, salmochelins, yersiniabactin and aerobactin [10,11]. The biosynthesis of the first three requires a 4′-phosphopantetheinyl transferase (PPTase). These enzymes activate polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) by catalyzing the transfer of a phosphopantetheinyl (P-pant) moiety from coenzyme A to conserved serine residues on PKSs and NRPSs [12,13]. In organisms containing multiple P-pant-requiring pathways, each pathway generally involves a dedicated cognate PPTase [12]. In *E. coli*, the EntD PPTase is involved in the synthesis of enterobactin [14] and salmochelins, which are glycosylated forms of enterobactin [15]. The IroA locus responsible for salmochelin production is located either on a chromosomal pathogenicity island or on a transmissible plasmid [16]. Contrary to enterobactin, salmochelins are able to evade the...
The synthesis of numerous molecules involved in the virulence potential and fitness of pathogenic bacteria requires a particular enzyme family, i.e. phosphopantetheinyl transferases (PPTases). To date, the synthesis of a given bioactive metabolite was thought to require a specific PPTase. As PPTases are being investigated as promising targets for antibacterial development, we addressed the question of a possible functional interchangability between PPTases in Escherichia coli. PPTases are known to be involved in the synthesis of low-molecular weight iron chelators (siderophores), and of a genotoxin named colibactin. Here we demonstrated interplay between the synthesis pathways leading to the production of siderophores and of colibactin. We showed that inactivation of both PPTases abolished the virulence of extra-intestinal pathogenic E. coli (ExPEC) in a mouse sepsis model. To our knowledge, this is the first demonstration of interplay between multiple PPTases-requiring pathways leading to the biosynthesis of functionally distinctive virulence factors, in a given microorganism. The extensive substrate specificity of PPTase ClbA could account for the co-selection and co-evolution of genomic islands encoding colibactin and yersiniabactin siderophore.

mammalian innate immune response protein lipocalin 2 (side-rocinal) and are therefore more potent virulence factors [17]. The other siderophore necessitating a PPTase is yersiniabactin. This siderophore is encoded by the high-pathogenicity island (HPI) that was acquired through horizontal transfer [18]. The HPI core region was detected in more than 70% of ExPEC isolated from blood cultures, urine samples and cerebrospinal fluid [19]. While yersiniabactin production in Yersinia requires the YbtD PPTase encoded outside the HPI [20], no gene homologous to ybtD has been identified in the genome of E. coli strains producing yersiniabactin. The PPTase committed to the synthesis of yersiniabactin in E. coli remains unknown.

We have shown that a number of E. coli strains from phylogenic group B2 display also the pks island, which codes for the production of colibactin, a polyketide-non ribosomal peptide genotoxin [21]. Colibactin is known to induce DNA double-strand breaks, cell cycle arrest in G2-phase and megalocytosis in infected eukaryotic cells [21]. E. coli strains harboring the pks island can induce DNA damage in enterocytes in vivo and trigger genomic instability in mammalian cells [22]. In a rodent model of colon inflammation, colibactin was demonstrated to potentiate the development of colon cancer [23]. Surprisingly, colibactin is also required for the colonic anti-inflammatory properties of the probiotic E. coli strain Nissle 1917 [24]. The synthesis of colibactin requires a PPTase encoded by the clbA gene located on the pks island [21]. Epidemiological studies revealed that the majority (73.1%) of the colibactin-positive E. coli strains were clinical ExPEC and that the pks island was significantly associated with a highly virulent subset of ExPEC isolates [25].

Strikingly, an analysis of the prevalence of the colibactin island among Enterobacteriaceae revealed that the pks island was constantly associated with the yersiniabactin gene cluster [26].

In this work we investigated a potential interplay between the biosynthetic pathways leading to the production of siderophores and of the colibactin genotoxin, through a possible functional interchangeability between PPTases in E. coli. We demonstrated that ClbA can contribute to the synthesis of siderophores both in vitro and in vivo. We proved in a mouse model of sepsis that the presence of either functional EntD or ClbA is required to maintain full virulence of ExPEC. This evidenced the interconnection between pathways leading to the synthesis of distinct secondary metabolites, via the PPTase ClbA. Therefore, the strict association of the pks island with HPI could have been selected in highly virulent E. coli isolates because ClbA can contribute to the synthesis of both the genotoxin and yersiniabactin.

**Results**

The pks island does not code for the biosynthesis of a siderophore in vitro

Because colibactin and siderophores belong to the same family of chemical compounds, we investigated first whether the pks island could not only allow the production of a genotoxin, but also of a siderophore. The entE gene, that encodes the ligase component of synthase multienzyme complex necessary for the enterobactin biosynthesis, was inactivated in the enterobactin producer E. coli strain MG1655. The resulting MG1655 entE mutant strain was shown not to produce any siderophore, as detected on CAS plate (Fig. 1A). The wild type (WT) and entE derivative of strain MG1655 were transformed with the bacterial artificial chromosome (BAC) harboring the entire pks island (BAC pks”). Both strains MG1655+BAC pks” and MG1655 entE+BAC pks” were shown to produce the genotoxin, as evidenced by the induction of double-strand breaks in eukaryotic cells (data not shown). The production of siderophore was qualitatively investigated in the resulting strains by plating on CAS plates (Fig. 1A). A yellow halo was not observed surrounding the bacterial colonies of MG1655 entE+BAC pks” (Fig. 1A). We proved in a mouse model of sepsis that the clbA gene was deleted. A yellow halo was produced around siderophore secreting bacteria.

Figure 1. Siderophore production by Escherichia coli strain MG1655 and derivatives. Chrome azurol S (CAS) plates upon which the E. coli strain MG1655 and derivatives have been grown overnight. A. Wild type and entE derivatives of strain MG1655. B. Wild type and entD derivatives of strain MG1655. BAC pks” is a bacterial artificial chromosome (BAC) harboring the entire pks island. BAC pksΔclbA is a BAC harboring the entire pks island where the clbA gene was deleted. A yellow halo is produced around siderophore secreting bacteria.

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strain MG1655 entE+BAC pks⁺. This showed that the pks island did not code for the biosynthesis of a siderophore.

**ClbA, the PPTase encoded on the pks island, can support the enterobactin siderophore synthesis in vitro**

In order to test whether the ClbA PPTase was functionally capable of participating to the biosynthesis of enterobactin, the entD gene was disrupted in *E. coli* strain MG1655. The resulting MG1655 entD mutant strain was subsequently transformed with BAC pks⁺ and with the BAC harboring the entire pks island where the clbA gene was deleted (BAC pksΔclbA). The production of siderophore was investigated by plating the resulting strains on CAS medium (Fig. 1B). This revealed that disruption of the entD gene in strain MG1655 resulted in the abrogation of the production of enterobactin (Fig. 1B). The introduction of the intact pks island in strain MG1655 entD restored the production of yellow pigment surrounding the colonies. This was not observed upon the introduction of the pks island disrupted for the clbA gene (Fig. 1B). Introduction of a functional plasmidic clbA gene in strain MG1655 entDΔBAC pksΔclbA and in strain MG1655 entD restored the production of enterobactin (data not shown).

These data evidenced that the ClbA PPTase can contribute to the enterobactin siderophore synthesis in vitro.

**Both EntD and ClbA can support the yersiniabactin siderophore synthesis in vitro**

Yersiniabactin is a siderophore the biosynthesis of which requires the PPTase YbtD in *Yersinia pestis* [20]. Although numerous *E. coli* strains were shown to produce yersiniabactin, an in silico analysis of the genome of all the *E. coli* strains available to date did not reveal any gene homologous to the ybtD gene.

In order to test whether the *E. coli* PPTase was functionally proficient to participate to the biosynthesis of yersiniabactin, we analyzed the enterobactin and yersiniabactin producer *E. coli* strain SE15. The entD gene was disrupted in *E. coli* strain SE15. The resulting SE15 entD mutant strain was subsequently transformed with plasmids carrying wild type entD gene or clbA gene. The production of total siderophores was qualitatively (Fig. 2A) and quantitatively (Fig. 2B) investigated using the CAS assay. This revealed that disruption of the entD gene in strain SE15 resulted in the abrogation of the production of siderophores (Fig. 2A and 2B). As expected, complementation with pEntD gene restored the production of siderophores. Remarkably, complementation with clbA gene also resulted in the synthesis of siderophores (Fig. 2A and 2B). The synthesis of yersiniabactin was specifically quantified in the different SE15 derivatives (Fig. 2C). This revealed that in the entD mutant, the yersiniabactin biosynthesis was abolished. The introduction of entD or clbA genes in SE15 entD mutant strain resulted in the restoration of yersiniabactin production.

These data showed that in *E. coli* strain SE15, EntD is the PPTase dedicated to the synthesis of yersiniabactin. Moreover, the EntD function can be substituted by ClbA. This suggests that both EntD and ClbA are involved in the synthesis of yersiniabactin in *E. coli* strains producing endogenously EntD and ClbA.

**Colibactin synthesis cannot be sustained by EntD in vitro**

As our data demonstrated that ClbA could complement EntD for the synthesis of enterobactin and yersiniabactin, we investigated whether EntD could rescue a clbA mutant for the production of colibactin. The entD gene was disrupted alone or in combination with the clbA gene in the colibactin producing *E. coli* strain M1/5. The M1/5 entD clbA double mutant was transformed with multicopy plasmids harboring wild type entD or clbA genes. The production of colibactin was quantified in the resulting strains through the quantification of megalocytic cells (Fig. 3A) and phosphorylation of H2AX histone (Fig. 3B) which correlate with DNA double strand breaks resulting from the genotoxic effect of colibactin [21,22].

HeLa cells were infected with the different strains for 4 hours, fixed and stained with methylene blue in order to quantify the megalocytosis effect, as previously described [21]. This revealed that the megalocytosis effect observed with the M1/5 entD mutant strain was similar to the effect measured with the wild type M1/5 strain (Fig. 3A). Inactivation of the clbA gene in the M1/5 entD mutant abrogated the colibactin effect (Fig. 3A). Transformation of the M1/5 entD clbA mutant strain with plasmids carrying the functional wild type clbA gene resulted in the restoration of the megalocytosis. A partial complementation of the double mutation was observed with plasmid p-clbA (1) whereas the double mutant was fully complemented with p-clbA (2). The different copy number of the plasmids can account for the quantitative differences observed below. A complementation was not observed when the wild type entD gene was expressed from a multicopy plasmid in the double mutant (Fig. 3A).

Genotoxicity of colibactin [21] was also examined in HeLa cells using H2AX assay based on indirect DNA double strand break detection using In Cell Western (ICW) with infrared fluorescence for H2AX phosphorylation (γ-H2AX) quantification [27]. HeLa cells were infected with strains M1/5, M1/5 entD, M1/5 entD clbA or M1/5 entD clbA complemented with entD. Following the quantification of the γ-H2AX (green) and the DNA (red) signals (Fig. 3B), respectively, the fold induction of γ-H2AX per cell was calculated. This revealed a genotoxic dose-response depending on the multiplicity of infection (MOI, Fig. 3B). No difference of γ-H2AX per cell was observed between WT and entD mutant strains. Infection of HeLa cells with mutant M1/5 entD clbA did not induce phosphorylation of H2AX. Moreover, the introduction of the functional entD gene did not result in the generation of DNA double strand breaks in strain M1/5 entD clbA (Fig. 3B).

Altogether, these data evidenced that EntD does not contribute to the colibactin synthesis, even when highly expressed on a multicopy plasmid.

**Colibactin synthesis can be sustained by exogenous PPTases in vitro**

We then investigated whether other PPTases, originated from other bacterial species, could rescue a clbA mutant for the production of colibactin. The clbA gene was disrupted in *E. coli* strain M1/5. The M1/5 clbA mutant was transformed with plasmids harboring wild type ybdD gene that encodes the YbdD PPTase in *Yersinia pestis*, pptT gene the PptT PPTase in *Mycobacterium tuberculosis*, sfp gene the Sfp PPTase in *Bacillus subtilis*, and clbA gene. PptT is involved in biosynthesis of the mycobactin siderophore [28] and is essential for mycobacterial viability [29]. Sfp is required for production of the peptide antibiotic surfactin [30]. The production of colibactin was quantified in the resulting strains through the quantification of megalocytic cells (Fig. 4A) and phosphorylation of H2AX histone (Fig. 4B). This revealed that both the megalocytosis and the H2AX phosphorylation were restored in the clbA mutant upon introduction of ybdD, pptT and sfp genes.

These data evidenced that ClbA can be xeno-complemented for the colibactin synthesis.
Figure 2. Both EntD and ClbA can support the yersiniabactin siderophore synthesis in vitro. Siderophore production by the enterobactin and yersiniabactin siderophores producer Escherichia coli strain SE15 and derivatives. A. Chrome azurol S (CAS) plate upon which E. coli strain SE15 and derivatives have been streaked for overnight growth. B. Quantification of total siderophore production in supernatants of E. coli strain SE15 and derivatives determined by the CAS assay. The data are the means and standard deviations of 5 independent experiments. C. Quantification of the yersiniabactin siderophore production in E. coli strain SE15 and derivatives. E. coli strains HB101, MG1655 and DH5α were used as negative controls (K12). RLU: relative light units. ***: P<0.001, **: P<0.01, *: P<0.05.

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ClbA is more promiscuous in its substrate specificity than EntD

In order to confirm that EntD and ClbA have narrow and broad substrate-specificity, respectively, we investigated whether EntD and ClbA had the capacity to activate the carrier protein involved in a reporter biosynthetic pathway. When activated by a PPTase, the single-module non-ribosomal peptide synthetase BpsA from *Streptomyces lavendulae* synthesizes a colored product (indigoindine), from a single substrate (L-glutamine) [31]. Plasmid p-BpsA that encodes BspA was transformed into strain MG1655 entD. The resulting MG1655 +p-entD strain was subsequently transformed with plasmids carrying ybtD, pptT, sfp, or entD genes. In addition, *E. coli* strain MG1655 BAC *pks* + and MG1655 BAC *pksAclth* were transformed with p-BpsA. The resulting strains that carry both the NRPS and a functional PPTase were grown in auto-induction medium, as previously described [32]. A blue coloration was detectable in cultures after overnight incubation for all strains but strain MG1655 +p-entD in MG1655 +p-pptT (Fig. 5A). A quantification of the indigoindine production was determined for all the strains (Fig. 5B). This confirmed that contrary to EntD, the PPTases YbtD, PptT, Sfp and ClbA were able to participate to the synthesis of the blue pigment.

This strengthens the fact that ClbA is more promiscuous in its substrate specificity than EntD in *E. coli*.

**Both EntD and ClbA must be inactivated to abolish virulence of ExPEC in a mouse model of sepsis**

In order to address the consequences, on the virulence of *E. coli*, of the cross talk between the synthesis pathways of colibactin and
siderophores demonstrated in vitro, we investigated *E. coli* strain SP15, an extra-intestinal pathogenic *E. coli* strain (ExPEC) of serotype O18:K1:H7 isolated from neonatal meningitis, in a mouse model of sepsis. *E. coli* strain SP15 produces colibactin and four different siderophores (aerobactin, yersiniabactin, enterobactin and salmochelin). The *entD* or *clbA* genes were disrupted individually and in combination. The strains were injected individually into the mice footpad; and the mice survival was monitored (Fig. 6A). This revealed that all the strains but SP15 *entD clbA* induced 70% mortality within 40 hours after injection. In contrast, virulence of strain SP15 *entD clbA* was completely attenuated in this mouse model of sepsis (Fig. 6A).

The bacterial dissemination in the mice was analyzed (Fig. 6B). Mice were sacrificed 18 hours post injection with PBS, WT strain, single or double mutants. Spleens and blood samples were collected, and bacteria were quantified by plating on selective medium (Fig. 6B). We observed that in both spleen and blood of infected animals the bacterial loads were similar with all the strains, but strain SP15 *entD clbA* induced 70% mortality within 40 hours after injection. In contrast, virulence of strain SP15 *entD clbA* was completely attenuated in this mouse model of sepsis (Fig. 6A).

The presence of either functional EntD or ClbA is required to maintain full virulence of ExPEC in a mouse model of sepsis.

In order to investigate the relative importance of EntD and ClbA in the virulence of *E. coli*, the SP15 *entD clbA* mutant strain was transformed with plasmids harboring *clbA* or *entD* functional genes. The resulting complemented strains were injected in mice (Fig. 7). This showed that complementation of strain SP15 *entD clbA* with either *clbA* or *entD* totally restored the virulence of the

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**Figure 4. Colibactin synthesis can be sustained by exogenous PPTases in vitro.** Colibactin production by *Escherichia coli* strain M1/5 and derivatives determined by megalocytosis (A) and by quantification of DNA double strand breaks (B), as in Fig. 3. ***: *P* < 0.001, **: *P* < 0.01, ns: not significant. *E. coli* strain SE15, which is devoid of colibactin locus, was used as a negative control. The *ybtD* gene encodes the YbtD PPTase in *Yersinia pestis*, the *pptT* gene the PptT PPTase in *Mycobacterium tuberculosis*, and the *sfp* gene the Sfp PPTase in *Bacillus subtilis*.

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strain (Fig. 7A). A slight but statistically significant delay in survival kinetics was observed when strain SP15
entD clbA complemented with the clbA gene was used for the injections (Fig. 7A). The quantification of bacteria in spleen and blood of the infected animals was determined (Fig. 7B). This revealed that complementation with clbA or entD allowed the survival of strain SP15
entD clbA in vivo, in a statistically significant manner at least in blood (Fig. 7B).

This evidenced that the presence of either functional EntD or ClbA is required to maintain full virulence of ExPEC in a mouse model of sepsis.

Discussion

Our work demonstrates the interplay between the biosynthetic pathways of a genotoxin and multiple siderophores. We have shown that ClbA, encoded by the pks island, is a promiscuous PPTase which promotes the synthesis of colibactin, yersiniabactin, enterobactin and consequently salmochelins. Although we demonstrated that ClbA could substitute for an entD mutation, the reciprocity was not observed. EntD seems to be specific for the synthesis of siderophores, which is consistent with other published reports [32]. In contrast, YbtD, the PPTase involved in yersiniabactin production in Yersinia was shown to substitute for a clbA mutation and allowed the production of colibactin. Attempts to relate conserved motifs of the group II subfamily of PPTases [12] with substrate specificity did not allow us to understand the functional promiscuity evidenced among certain PPTases, since type II PPTases usually have very remote primary sequences. Unfortunately, it is not possible to compare either the 3D structure of these PPTases because only the structure of Sfp is available [33]. Type II PPTases are predicted to have a similar folding and very similar secondary structures [33]. However it is difficult to draw conclusions on the folding of proteins and to correlate it with substrate specificity. Only the comparison of 3D high-resolution structures would provide information about the structure/function relationship of PPTases. Our work provides novel evidence that make PPTases promising targets for antibacterial development [34], because these enzymes are crucial for the biosynthesis of a multitude of a pathogen’s collection of essential metabolites and virulence factors [35].

Iron is an essential element for survival of E. coli. Therefore, E. coli strains have evolved a strategy for iron acquisition which uses multiple siderophores with high-affinity for ferric iron. These include enterobactin, salmochelins, aerobactin and yersiniabactin [11]. Each siderophore has specific affinity for iron and may be differentially regulated to provide different advantages, potentially allowing extra-intestinal pathogenic E. coli (ExPEC) to adapt to different environmental conditions or to overcome host innate immunity [10,36,37]. In our model of sepsis, the ExPEC mutant that produced only aerobactin as a siderophore (strain SP15
entD clbA) was completely attenuated. This suggests that aerobactin plays a minor role in the iron uptake in this sepsis model; but the importance of each siderophore can be host and strain dependent [38]. Interestingly, either ClbA or EntD were able to restore the virulence of strain SP15
entD clbA. However, we have shown that colibactin synthesis cannot be sustained by EntD. This suggests that not colibactin, but the siderophore systems (alone or in combination) are critical during the first step of the infection in this strain (Fig. 7A). A slight but statistically significant delay in survival kinetics was observed when strain SP15
entD clbA complemented with the clbA gene was used for the injections (Fig. 7A). The quantification of bacteria in spleen and blood of the infected animals was determined (Fig. 7B). This revealed that complementation with clbA or entD allowed the survival of strain SP15
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entD clbA. However, we have shown that colibactin synthesis cannot be sustained by EntD. This suggests that not colibactin, but the siderophore systems (alone or in combination) are critical during the first step of the infection in this
mouse model of sepsis. Indeed, the bacterial loads in both spleen and blood were similar in animal infected with SP15 entD clbA mutant complemented either with ClbA or EntD. Analysis of bacteria present in the popliteal lymph node confirmed this analysis (data not shown). Since the carriage of the pks island is correlated with successful long-term gut colonization in humans [39], colibactin could be important for the commensal lifestyle of ExPEC. Moreover, our unpublished data suggest that the genotoxin colibactin could also play a role in natural sepsis since lymphocytes are susceptible to the genotoxin.

Phylogroup B2, which includes the majority of ExPEC isolates, is considered to represent the evolutionary eldest lineage within the species [40]. Interestingly, the pks island found in B2 isolates is highly conserved, and is physically associated to a highly conserved High-Pathogenicity Island. This might even point towards a recent emergence of a distinct subgroup within phylogroup B2. In fact, epidemiological knowledge allows defining specific clonal lineages with high ExPEC virulence potential [41]. We believe that the most virulent and also the best colonizer of human gut resulted from a step-by-step acquisition and selection of different mobile elements. We propose here a scenario with the sequential integration of at least two pathogenicity islands and the cross talk via two PPTases (Fig. 8). At first, all E. coli strains produce at least one siderophore i.e. enterobactin. The entD gene and the other genes of the enterobactin system are part of the core genome and have been identified in all the E. coli strains isolated so far [42]. In contrast, the HPI encoding the yersiniabactin siderophore system devoid of any PPTase gene was acquired by horizontal gene transfer. Almost all E. coli HPIs appear to result from a single ancestor, which entered the E. coli species rather recently [43]. All strains of the phylogenetic group B2 and almost all of group D carry the HPI, whereas strains of groups A and B1 were found to be only occasionally HPI positive (Fig. 8, [19]). The spread of the HPI must have occurred in a dramatically fast fashion, which may indicate a strong selective pressure. We have shown in this study that EntD is actually the PPTase that mediates the synthesis of a functional yersiniabactin. E. coli strains that contain the HPI were demonstrated to be more virulent than isolates that lack the island [18]. Moreover, yersiniabactin is frequently associated with urinary tract infections [44,45]. The pks island is known to be confined to the phylogenetic group B2. Besides, the pks island is highly represented within an especially highly virulent subset of B2 strains that exhibit extremely elevated virulence scores and an increased likelihood of causing bacteremia [25]. It has been previously demonstrated that all the E. coli strains that acquired the pks island encoding the colibactin through horizontal transfer, also displayed the HPI locus, with an integration site in tRNA asnW gene and asnT gene, respectively (Fig. 8; [26]). The pks island appears to be highly conserved (or even identical) in terms of nucleotide sequence in different E. coli isolates [26]. This may be a hint to a more recent acquisition of the pks island, compared to the HPI, which displays about 1–2% sequence divergence among the E. coli isolates (Schubert, unpublished data). We hypothesize that the association of the pks island with HPI has been selected in the highly virulent E. coli isolates because ClbA can contribute to the synthesis of both the genotoxin and yersiniabactin (and also

Figure 6. Both EntD and ClbA must be inactivated to abolish virulence of ExPEC. Mice underwent footpad injection with 10^8 CFU of E. coli SP15 wild type strain or derivatives. A. The percentage of mice survival was monitored. 10 to 25 mice were used per group. B. 18 h post infection 4 to 10 mice per group were sacrificed. Bacteria were quantified in spleen and blood collected from each animal. For statistical analysis, two-factor ANOVA and Bonferroni’s multiple comparison test was performed. ***: P<0.001, **: P<0.01.

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enterobactin and consequently salmochelins). This deadly association is not confined in *E. coli*. Similar events also occurred in other pathogenic *Enterobacteriaceae* since the *pks* island was also detected in *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Citrobacter koseri* isolates where the island is also physically associated on the chromosome with the HPI locus [26].

**Materials and Methods**

**Bacterial strains, mutagenesis procedures and growth conditions**

Bacterial strains used in this study are listed in Table 1. *E. coli* SE15 (O150:H5) is a human commensal bacterium isolated from feces of a healthy adult and classified into *E. coli* phylogenetic group B2 [46]. Strain SE15 is devoid of the *pks* island. *E. coli* M1/5 is a human commensal bacterium isolated from feces of a healthy adult and classified into *E. coli* phylogenetic group B2. Strain M1/5 harbors of the *pks* island. Strain SP15 is an extra-intestinal pathogenic *E. coli* strain (ExPEC) of serotype O18:K1:H7 isolated from neonatal meningitis. Strain SP15 harbors the *pks* island. The repertoire of siderophores the *E. coli* strains possess is indicated in Table 1. Gene inactivations were engineered by using the lambda Red recombinase method [47] using primers listed in Table 2. For complementation, the *clbA* gene was cloned into plasmid pASK75, a cloning vector that harbors a ColE1 origin of replication and therefore is low copy number plasmid (p-*clbA* (1), table 1) or PCR-Script, a cloning vector that harbors a ColE1 origin of replication and therefore is high copy number plasmid (p-*clbA* (2), table 1). For complementation, the *entD* gene was cloned into PCR-Script (p-*entD*, table 1).

Before injection to mice, all *E. coli* strains were grown overnight in LB broth supplemented with antibiotics if required, at 37°C with shaking. These cultures were diluted 1:100 in LB broth with antibiotics when necessary and grown for 3 h at 37°C with shaking. Bacterial cells were resuspended in sterile PBS to the appropriate concentration (2×10^9 CFU/mL). All the strains were shown to display similar growth kinetics *in vitro* in LB broth (data not shown).

**Detection and quantification of total siderophores**

Chrome azurol S (CAS) assay was used to detect siderophores produced by *E. coli*. The CAS solution was prepared according to Schwyn and Neilands [48]. *E. coli* strains were grown on CAS agar plates and incubated at 37°C overnight in the dark. The colonies with orange zones were siderophore-producing strains [48]. To quantify siderophore synthesis, 500 μL of CAS indicator solution containing 4 mM sulfosalicylic acid was mixed with the same volume of supernatant. The reaction mixtures were incubated for 60 min at room temperature to allow complex formation, and the siderophore-dependent color change was determined at OD630 nm. For quantification, the iron chelating agent 8 hydroxyquinoline (8HQ, sigma-aldrich) was used as the standard.
Figure 8. Model for the co-evolution of the \textit{pks} and high pathogenicity islands in \textit{E. coli}. Left. Phylogenetic relationships amongst the \textit{E. coli} reference strains (ECOR, [3,52]). The phylogeny was based on MLST of back-bone genes [3,43]. Ent locus positive strains are indicated in blue, HPI island positive strains are indicated in green [43], iro locus positive strains are indicated in orange and \textit{pks} island positive strains are indicated in pink [21]. The presence of the iro locus was determined only in B2 strains. Right. The archetypal chromosome of phylogroup B2 \textit{E. coli} strains. The loci encoding enterobactin (ent), yersiniabactin (HPI), salmochelin (iro) and colibactin (pks) are represented. The arrows originating from PPTases EntD and ClbA and pointing towards other loci illustrate the capacity of the PPTase to contribute to the synthesis of metabolites from other loci.

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Table 1. Strains and plasmids used in the study.

| Strain or plasmid | Genotype or phenotype | Source or reference |
|-------------------|-----------------------|---------------------|
| E. coli strains   |                       |                     |
| DH10B             | Enterobactin siderophore producer |                     |
| DH5α              | Enterobactin siderophore producer |                     |
| HB101             | Enterobactin siderophore producer |                     |
| MG1655            | Enterobactin siderophore producer |                     |
| WR1542+pACYC5.3L  | Tc’, Ap’, Kan’, Cm’; fepA::Tn10dTc; iroCN::Gmr; cir::MudI carrying pACYC5.3L plasmid | Gift from W. Rabbsch |
| MG1655 entE       | entE mutant of strain MG1655; Kan’ | This study |
| MG1655 entE+entD  | entE mutant strain MG1655 carrying BAC entD; Kan’, Cm’ | This study |
| MG1655 entD       | entD mutant strain MG1655; Kan’ | This study |
| MG1655 entD+BAC pks+ | entD mutant strain MG1655 carrying BAC pks+; Kan’, Cm’ | This study |
| MG1655 entD+p-cblA (1) | entD mutant strain MG1655 carrying p-cblA (1); Kan’ Amp’ | This study |
| MG1655 ΔentD      | entD mutant strain MG1655 | This study |
| MG1655 ΔentD+p-cblA | entD mutant of strain MG1655 carrying p-cblA; Kan’ | This study |
| MG1655 ΔentD+p-entD | entD mutant strain MG1655 carrying p-entD; Kan’ Amp’ | This study |
| MG1655 ΔentD+p-cblA (1) | entD mutant strain MG1655 carrying p-cblA (1); Kan’ Amp’ | This study |
| MG1655 ΔentD+p-sfp | entD mutant of strain MG1655 carrying p-sfp; Kan’ Amp’ | This study |
| MG1655 ΔentD+p-pptT | entD mutant of strain MG1655 carrying p-pptT; Kan’ Amp’ | This study |
| MG1655Δ+BAC pks+  | strain MG1655 carrying BAC pks+; Cm’ | This study |
| MG1655Δ+BAC pks+p-entD | mutant of strain MG1655 carrying p-entD; Kan’ Amp’ | This study |
| MG1655Δ+BAC pks+p-sfp | mutant of strain MG1655 carrying p-sfp; Kan’ Amp’ | This study |
| MG1655Δ+BAC pks+p-cblA | mutant of strain MG1655 carrying p-cblA (1); Kan’ Amp’ | This study |
| MG1655Δ+BAC pks+p-cblA | mutant of strain MG1655 carrying p-cblA; Kan’ | This study |
| SE15 entD         | entD mutant of strain SE15; Kan’ | This study |
| SE15 entD+p-entD  | entD mutant of strain SE15 carrying p-entD plasmid; Kan’ Amp’ | This study |
| SE15 entD+p-cblA (1) | entD mutant of strain SE15 carrying p-cblA (1) plasmid; Kan’ Amp’ | This study |
| M1/S entD         | entD mutant of strain M1/S; Kan’ | This study |
| M1/S cblA         | cblA mutant of strain M1/S; Kan’ | This study |
| M1/S entD cblA    | cblA mutant of strain M1/S; Kan’ | This study |
| M1/S entD cblA+entD | cblA mutant of strain M1/S carrying p-entD; Kan’ Amp’ | This study |
| M1/S entD cblA+entD (1) | cblA mutant of strain M1/S carrying p-cblA (1); Kan’ Amp’ | This study |
| M1/S entD cblA+p-cblA (2) | cblA mutant of strain M1/S carrying p-cblA (2); Kan’ Amp’ | This study |
| M1/S entD cblA+p-cblA (1) | cblA mutant of strain M1/S carrying p-cblA (1); Kan’ Amp’ | This study |
| M1/S cblA+p-sfp   | cblA mutant of strain M1/S carrying p-sfp plasmid; Kan’ Amp’ | This study |
| M1/S cblA+p-pptT  | cblA mutant of strain M1/S carrying p-pptT plasmid; Kan’ Amp’ | This study |
| SP15 entD         | entD mutant of strain SP15; Kan’ | This study |
| SP15 cblA         | cblA mutant of strain SP15; Kan’ | This study |
| SP15 entD         | entD mutant of strain SP15; Kan’ | This study |
| SP15 cblA         | cblA mutant of strain SP15; Kan’ | This study |
| SP15 ΔentD cblA+entD | cblA mutant of strain SP15 carrying p-entD; Kan’ Amp’ | This study |
| Plasmids          |                       |                     |
| pACYC5.3L         | fyuA-, ybtA-, yfu-luc-, ip6-8, Cm’ | Gift from W. Rabbsch |
| p-entD            | High copy number PCR-Script plasmid carrying entD gene; Amp’ | This study |
| p-cblA (1)        | Low copy number pASK75 plasmid carrying cblA gene; Amp’ | Gift from U. Dobrindt |
| p-cblA (2)        | pMB808, high copy number PCR-Script plasmid carrying cblA gene; Amp’ | [22] |
| BAC pks+          | Bacterial artificial chromosome carrying the entire pks island; Cm’ | [21] |
| BAC pksΔcblA      | Bacterial artificial chromosome carrying the entire pks island with deleted cblA gene; Cm’ | [22] |
Quantification of yersiniabactin

The expression of the *fyuA* gene encoding the yersiniabactin receptor (FyuA) is known to be up-regulated in the presence of extracellular yersiniabactin [49]. Thus, yersiniabactin-dependent up-regulation of *fyuA* expression can be monitored by means of a *fyuA*-reporter fusion in the indicator strain [50].

Bacterial strains were cultivated in NBD medium, i.e. Nutrient Broth (NB) medium supplemented with 200 μM 2,3′-dipyridyl (Sigma), for 24 h at 37°C. Bacteria were pelleted by centrifugation and the supernatant was added to the indicator strain WR1542 (Sigma), for 24 h at 37°C. Determination of the genotoxic effect induced by colibactin

**Detection and quantification of indigoidine**

After overnight cultures in LB broth supplemented with the appropriate antibiotics, bacteria were diluted 1:10 in M9 minimal medium supplemented with 100 mM L-glutamine and 1 mM IPTG, and cultivated 16 h at 18–20°C under shaking [31,51].

Bacteria were then collected by centrifugation at 900× g for 5 min. At this speed, bacterial cells were pelleted while indigoidine still remained in the supernatant [31]. Indigoidine production was quantified by measuring the absorbance of blue-colored supernatant (OD_{612 nm}). The bacterial pellet was resuspended in PBS, and biomass was quantified by measuring the absorbance (OD_{630 nm}). Finally, the indigoidine production was normalized with the ratio Indigoidine/Biomass (e.g. ratio OD_{612 nm}/OD_{630 nm}).

**Table 2. Oligonucleotides used in the study.**

| Primers | Sequences |
|---------|-----------|
| entD-P1 | GGCGCGATCGTGCACATTTATTGGCCTGGGAAATATGGCATTAATAGTTGAGCTGTAAGCCAGAGCTGCTTC |
| entD-P2 | TCAGTTTCATATGACCTCCTTGGCGGAAGTAGCAGTGCAGCGCCGATGAAATATCCCTTCTAG |
| entD-Up | CCCCCGGGAGGAGCTGAGTATATAGGAG |
| entD-Down | AACCTCGAAGACGACCTGCTTTATTACCTTC |
| entE-P1 | TATCGACGCGGACGACAGTGGATATCACGGGAGGTAGGCGGCTGAGCAGCTGCTTC |
| entE-P2 | AAGCGGACCTTTTGCCGACATGCTCCTCAATCCCTGCTCACGGCCGATGAAATATCCCTTCTAG |
| JPN2 | CAG ATA CAC AGA TAC CAT TCA |
| JPN46 | CTA GAT TAT CCG TGG CGA TTC |

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Belgium) supplemented with phosphatase inhibitor PHOSTOP (Roche), followed by overnight incubation with rabbit monoclonal anti γ-H2AX (Cell Signaling) (1:200). An infrared fluorescent secondary antibody absorbing at 800 nm (IRDye™ 900CW, Rockland) was then applied (dilution 1:500). For DNA labeling, RedDot2 (Biotium) was used (dilution 1:500) together with the secondary antibody. The DNA and the γ-H2AX were simultaneously visualized using an Odyssey Infrared Imaging Scanner (Li-Cor ScienceTec, Les Ulis, France) with the 680 nm fluorophore (red color) and the 800 nm fluorophore (green dye). Relative fluorescence units from the scanning allowed a quantitative analysis. Relative fluorescent units for γ-H2AX per cell (as determined by γ-H2AX divided by DNA content) were divided by vehicle controls to determine percent change in phosphorylation of H2AX levels relative to control. All experiments were carried out in triplicate.

Mouse sepsis model

Animal experiments were carried out in accordance with the European directive for the protection of animals used for scientific purposes. The protocols were validated by the local ethics committee on animal experiment “Comité d’éthique Midi Pyrénées pour l’expérimentation animale” which is affiliated to “Comité National de Réflexion Éthique sur l’Expérimentation Animale” and linked to the french ministry of research (Referenced protocols: PX-ANI-A2-94, 95, 96, 99, 100, and 101). Nine week old female C57BL/6 mice [JANVIER] were injected into the footpad with 10^8 ExPEC WT, mutant and clbA mutant complemented with p-entD, andentD +p-entD, together with intraperitoneal injection of 100 μL of carbenicillin (1.6 mg/mL) or PBS. When required, mice were sacrificed by lethal anaesthesia (rompun/ketamine in 0.9% NaCl) 18 h post injection. The abdominal cavity of anesthetized mouse was opened. The widest part of the posterior vena cava was localized and sectioned. Blood was collected by aspiration from the abdominal cavity. Spleens were surgically removed. Bacteria located in spleen cells were isolated from the mechanical dissociation of the splenic tissue using Precellys tissue homogenizer. Bacteria were quantified by plating of serial dilutions of blood and dissociated spleen on appropriate selective MacConkey agar. The antibiotics used to supplement the medium correspond to the resistance displayed by the different strains and are indicated in Table 1.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5.0d. The mean ± standard deviation (SD) is shown in figures, and P values were calculated using a one-way or two-way ANOVA followed by a Bonferroni post-test unless otherwise stated. For bacterial quantification, CFU by mg of spleen or mL of blood were log transformed for the analysis. A P value of less than 0.03 was considered statistically significant and is denoted by *, P<0.01 is denoted by ** and P<0.001 by ***.

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Author Contributions

Conceived and designed the experiments: PM IM JPN EO. Performed the experiments: PM IM GM MP CG DP MB MO. Analyzed the data: PM GM MO MA JPN SS EO. Contributed reagents/materials/analysis tools: MA CC. Wrote the paper: PM EO.

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