Aus dem
Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie
der Ludwig-Maximilians-Universität München
Lehrstuhl für Medizinische Mikrobiologie und Krankenhaushygiene
Direktor: Prof. Dr. med. Sebastian Suerbaum

Bedeutung der Siderophorsysteme für die Pathogenität der extraintestinal pathogenen *Escherichia coli*

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Giuseppe Magistro
aus
Fürstenfeldbruck
2018

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	Prof. Dr. med. Sören Schubert
Mitberichterstatter:	Prof. Dr. med. Franz Worek Prof. Dr. med. Christian Gratzke PD Dr. med. Claudius Füllhase
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Tag der mündlichen Prüfung:	22.03.2018

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Abkürzungsverzeichnis

E. coli = *Escherichia coli*
APEC = avian pathogenic *E. coli*
EAEC = enteroaggregative *E. coli*
EHEC = enterohämorrhagische *E. coli*
EIEC = enteroinvasive *E. coli*
EPEC = enteropathogene *E. coli*
ETEC = enterotoxische *E. coli*
ExPEC = extraintestinale pathogene *E. coli*
DAEC = diffus adhärente E. coli
HPI = High-Pathogenicity Island
IPEC = intestinal pathogene *E. coli*
K_d = Dissoziationskonstante
MNEC = Meningitis assoziierte *E. coli*
MLEE = Multilocus Enzyme Electrophoresis
MLST = Multilocus Sequence Typing
PPTase = Phosphopantetheinyl-Transferase
UPEC = uropathogene *E. coli*

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1. Einleitung

1.1. Die Spezies *Escherichia coli*

Der Pädiater und bedeutende Bakteriologe Theodor Escherich präsentierte im Jahr 1885 seine Forschungsarbeit über die Morphologie und Eigenschaften einer Population von Darmbakterien aus Neugeborenen und Säuglingen, die er als „*Bacterium coli commune*“ bezeichnete (1). Im Jahr 1919 wurde die Spezies ihm zu Ehren in *Escherichia coli* (*E. coli*) umbenannt. Die Bakterienspezies *E. coli* aus der Familie der *Enterobacteriaceae* stellt in vielerlei Hinsicht eines der bedeutendsten Bakterien für die Naturwissenschaft überhaupt dar. Neben seiner herausragenden Funktion als Modellorganismus für die naturwissenschaftliche Forschung werden die zahlreichen Facetten dieses faszinierenden Bakteriums durch seine medizinische Bedeutung in besonderem Maße hervorgehoben (2). Als gramnegatives, sporenloses, fakultativ anaerobes Stäbchenbakterium kolonisieren *E. coli* als Kommensale den menschlichen Gastrointestinaltrakt bereits wenige Stunden nach Geburt (3-5). Die bevorzugte intestinale Nische entspricht der oberen muzinösen Schicht von Colon und Zökum, wo es sich aufgrund seiner vermeintlich überlegenen Fähigkeit zur Utilisation von Kohlenhydraten, wie zum Beispiel Glukonat, zu einer der dominierenden Spezies in diesem Habitat etabliert hat (6, 7).

Im Rahmen der evolutiven Diversifizierung wurden hochspezialisierte Vertreter selektioniert, die es *E. coli* erlaubten sich an alternative Lebensräume mit speziellen Umweltbedingungen zu adaptieren. Dabei erlangten sie pathogenes Potenzial. Ein bestimmtes Armamentarium von hocheffektiven Virulenz- und Fitnessfaktoren und deren komplexe Koordination befähigt pathogene *E. coli* Infektionen innerhalb und außerhalb des Darmes zu verursachen. Das umfasst intestinal Infektionen und Durchfallerkrankungen, Harnwegsinfektionen, Pneumonien, operative Wundinfektionen, bakterielle Peritonitis, Neugeborenen-Meningitis und septische Krankheitsbilder (8). Klinisch lässt sich die Spezies somit nach dem entsprechenden Pathotypus in drei Kategorien unterscheiden: kommensale, intestinal pathogene (enterisch/diarrhoegene) und extraintestinale pathogene *E. coli* (9-11). Zu den anerkannten Untergruppen mit intestinaler Pathogenität (intestinal pathogene *E. coli*; IPEC) zählen enteropathogene *E. coli* (EPEC), enterohämorrhagische *E. coli* (EHEC), enterotoxinogene oder enterotoxische *E. coli* (ETEC), enteroaggregative *E.*

coli (EAEC) enteroinvasive *E. coli* (EIEC) und diffus adhärente *E. coli* (DAEC). Extraintestinale pathogene *E. coli* (ExPEC) sind Bestandteil des natürlichen intestinalen Mikrobioms und scheinen hier nicht Infektionen zu verursachen, jedoch besitzen sie die Fähigkeit Nischen außerhalb des Darmtraktes zu besiedeln und dort Infektionen auszulösen (11). Die häufigste extraintestinale Infektion entsteht durch uropathogene *E. coli* (UPEC) als ursächlicher Erreger der Harnwegsinfektion. In etwa 75% der unkomplizierten und 65 % der komplizierten Harnwegsinfektion werden UPEC isoliert (12). Als zweithäufigste Ursache für die neugeborenen Meningitis einhergehend mit einer hohen Mortalitätsrate von 20-29 % werden Meningitis assoziierte *E. coli* (MNEC) festgestellt (13).

1.2. Extraintestinal pathogene *Escherichia coli* (ExPEC)

Es stellt sich die Frage, was konkret die extraintestinale Pathogenität im Vergleich zu IPEC und kommensalen *E. coli* begründet. Ein Ansatz zur Beantwortung dieser Fragestellung basiert auf Typisierungstechniken (5, 14). Einer der ältesten Methoden besteht in der Serotypisierung anhand der Kombination von Oberflächenstrukturen wie dem O-Antigen (Lipopolysaccharid; 173 Antigene), dem K-Antigen (Kapsel; 80 Antigen) und dem H-Antigen (Flagellen; 56 Antigene) (15, 16). Hierdurch konnte erstmals die klonale Struktur der *E. coli* Population aufgedeckt werden. In den 1980er konnte durch *Multilocus Enzyme Electrophoresis* (MLEE) aufgezeigt werden, dass für die Spezies *E. coli* bei hoher genetischer Diversität nur wenige bestimmte Genotypen bestehen, was die Klassifizierung gemäß phylogenetischen Kriterien in die Gruppen A, B1, B2, D und E etablierte (17). Demnach sind ExPEC hauptsächlich in der Phylogruppe B2 und in geringerem Maße in Phylogruppe D zu finden, während IPEC und Kommensale meist in die Gruppen A und B1 eingeteilt werden (18, 19). Die aktuelle Referenzmethode stellt das *Multilocus Sequence Typing* (MLST) dar, welches anhand der Nukleotidsequenz von ausgewählten *housekeeping*-Genen eine Typisierung im Rahmen von populationsgenetischen Untersuchungen erlaubt (20). Mehrere Schemata sind je nach Selektion der *housekeeping*-Gene für *E. coli* etabliert (21-23). Die neueste Errungenschaft auf dem Gebiet der Populationsgenetik stellt die Entwicklung neuer *Next-Generation-Sequencing* Technologien dar, womit nun komplette Genome entschlüsselt werden (24, 25). Mittlerweile sind eine Vielzahl von Genomen unterschiedlicher *E. coli* Isolate sequenziert und annotiert worden (26-

30). Im direkten Vergleich zwischen apathogenen Erregern und pathogenen Bakterien wird angestrebt, eindeutige genetische Unterschiede zu bestimmen und somit Determinanten der Pathogenität zu definieren. Nach Sequenzierung von 20 Genomen und komparativer Analyse wurde ein Kern-Genom von ca. 2000 Gene in *E. coli* identifiziert, also der in allen Isolaten anzutreffende konservierte Genpool, wobei das korrigierte Pan-Genom, d.h. alle in *E. coli* identifizierten kodierenden Gene, auf etwas über 10000 Gene bestimmt wurde. Das durchschnittliche *E. coli* Genom beläuft sich auf 4721 Gene (27). Im Zuge weiterer globaler Genomanalysen zeichnet es sich immer deutlicher ab, dass die Plastizität des Genoms eine Vielzahl von Adaptationslösungen für eine bestimmte Nische offenbart und nicht ein definiertes Repertoire an Virulenz - und Fitnessgenen für extraintestinale Pathogenität existiert, geschweige denn „das“ exklusive ExPEC-spezifische Gen (5, 10). Diese genetische Flexibilität durch stetigen Fluss von Gewinn und Verlust genetischer Information lässt eine Vielfalt an Lösungen zu, um pathogenen als auch apathogenen Bakterien eine optimierte Anpassung an ein spezielles Habitat zu ermöglichen.

Die treibende Kraft der mikrobiellen Evolution stellen allgemein Mutationen, Insertionen, Deletionen und genomische Rearrangements dar. Unter den vielfältigen Mechanismen neue Eigenschaften innerhalb einer Spezies oder über die Speziesbarriere hinweg zu übertragen, hat der horizontale Gentransfer von sogenannten genomischen Inseln als besonders effektive Option große Aufmerksamkeit erlangt (31-34). Die Analyse dieser genomischen Inseln identifizierte einen gemeinsamen Bauplan, der folgende strukturellen Charakteristika enthält: (i) Es handelt sich um ausgedehnte genomische Bereiche einer Größe von 10 Kilobasen bis zu 200 Kilobasen, (ii) sie weisen einen G+C Gehalt auf, der sich vom Kerngenom signifikant unterscheidet, (iii) sie sind oftmals assoziiert mit Genen, die für Transfer-RNA kodieren, (iv) sie sind relativ instabil, (v) enthalten mobile genetische Elemente wie beispielsweise Insertionssequenzen, Transposons, Integrasen oder Bakteriophagen DNA, (vi) sie werden flankiert von repetitiven Sequenzen (*direct repeats*) und (vii) sie tragen pathogenspezifische Virulenzgene. Wird durch den Erwerb von Faktoren, die auf einer genomischen Insel lokalisiert sind, die Pathogenität des Erregers gesteigert, so bezeichnet man sie auch als Pathogenitätsinseln. Optimierte Fähigkeiten, um sich in einer bestimmen Nische erfolgreicher behaupten zu können, werden durch Fitnessfaktoren vermittelt.

Resultiert aus Verlust oder Inaktivierung eines Gens in pathogenen Bakterien eine signifikanten Reduktion von Virulenz beziehungsweise Pathogenität, die durch Restitution dieser Genfunktion wieder reversibel ist, so spricht man gemäß dem Koch'schen Postulat von Virulenzfaktoren oder Pathogenitätsfaktoren (35). Das Armamentarium der ExPEC an Virulenzfaktoren ist sehr vielseitig und umfasst Adäsine, Invasine, Toxine, Protektine, Eisenaufnahmesysteme, Autotransporter, Sekretionssysteme, Flagellen und Lipopolysaccharid (9, 36, 37).

1.3. Pathomechanismen: Deckung des Eisenbedarfs in ExPEC

Die Deckung des Eisenbedarfs ist dabei einer der essentiellsten Aufgaben, da bis auf wenige Ausnahmen (Laktobazillen, *Borrelia burgdoferi*, *Treponema pallidum*) Bakterien ohne Eisen nicht lebensfähig sind (38). Die Eisenhomöostase ist ein zentraler physiologischer Schlüsselpunkt für Säugetiere und Mikroorganismen zugleich (39, 40). Im menschlichen Organismus ist Eisen zentrale Komponente in Haemgruppen, Eisen-Schwefel-Cluster einer Vielzahl von Enzymen, wesentlicher Bestandteil der Atmungskette und DNA-Synthese. Im Rahmen von immunologischen Prozessen spielt Eisen eine entscheidende Rolle bei der Generierung von reaktiven Sauerstoffradikalen in Neutrophilen und Makrophagen und ist zudem beteiligt an der klonalen T-Zell Expansion (41). Der Gesamtbestand an Eisen im menschlichen Organismus beträgt ca. 4 bis 5 Gramm, wobei etwa 60 % bis 70 % davon in Hämoglobin gebunden vorliegen. Die Hämoglobinsynthese beansprucht in etwa 80 % des Eisenbedarfs (39). Aufgrund der Toxizität von freiem Eisen, was durch die *Fenton-Reaktion* zur Bildung von freien Sauerstoffradikalen führt, resultierend in der potentiell zellschädigenden Wirkung durch Proteindenaturierung, DNA-Strangbrüchen oder Lipidperoxidation, liegt es vornehmlich in gebundener Form vor in Transferrin, Laktoferrin, Ferritin oder komplexiert in Haemproteinen (40). Diese strikte Eisenhomöostase wird daher auch an sich als antimikrobieller Abwehrmechanismus angesehen (*nutritional immunity*), denn die benötigte Konzentration an Eisen für replizierende Mikroorganismen (ca. 10^{-6} bis 10^{-7} M) liegt etwa 11 bis 12 Logstufen höher als es physiologischerweise im Menschen frei verfügbar ist (ca. 10^{-18} M) (38, 40). Um dieser Entzugsstrategie zu begegnen, haben nun pathogene Bakterien ein umfassendes Spektrum an vielseitigen Eisenaufnahmesystemen entwickelt (38, 41-45). Diese erlauben über spezielle

Transportsysteme zum Beispiel zweiwertiges Eisen oder eisenbeladene Haemproteine zu verwerten.
Besonders erfolgreich ist in diesem Kontext die Sekretion von sogenannten Siderophoren (griechisch für Eisenträger). Es handelt sich dabei um niedermolekulare Verbindungen, die mit höchster Affinität gebundenes dreiwertiges Eisen dem Wirt entreißen und somit den Bakterien über spezifische Transportsysteme zur Verfügung stellen. ExPEC sind grundsätzlich in der Lage vier verschiedene Siderophore zu produzieren, die sich aufgrund ihrer chemischen Struktur in ihrer Bindungsaffinität für dreiwertiges Eisen unterscheiden (46, 47). Jedes Siderophor-System umfasst (i) einen Syntheseapparat, (II) ein spezielles Exportsystem, (iii) spezifische Außenmembranrezeptoren, um die beladenen Siderophore hochselektiv zu binden, (iv) einen Internalisierungspfad und schließlich (v) die Möglichkeit zur Freisetzung des Eisens aus dem Siderophor im Zytoplasma des Bakteriums. Auffällig ist, dass zusätzliche Rezeptoren für alternative Siderophore, die selbst nicht synthetisiert werden können, an der Außenmembran exprimiert werden. Durch diese Form der *Piraterie* profitieren Bakterien im direkten mikrobiellen Konkurrenzkampf um Nährstoffe.

1.3.1. Enterobactin

Enterobactin ist ein Siderophor, das nach seiner chemischen Struktur den Katecholaten zugeordnet wird (43, 46, 48). Es ist weit verbreitet unter pathogenen enteralen Bakterien, wird von fast allen *E. coli* Stämmen synthetisiert und wird in Isolaten von *Salmonella* spp*., Klebsiella* spp. und *Shigella* spp*.* detektiert (43, 48-50). Mit einer Dissoziationskonstante K_d von 10^{-52} M präsentiert es einen der stärksten natürlichen Eisenchelatoren überhaupt (51, 52). Das funktionelle Enterobactin-Gencluster besteht aus 14 Genen, die in 6 Operons organisiert sind (48). Auf diese höchst effektive Strategie reagieren interessanterweise Neutrophile im Rahmen der angeborenen Immunität mit der Produktion von Lipocalin-2, einem Molekül, das Enterobactin zu binden vermag und damit die Eisenaufnahme über dieses Siderophor verhindert (53, 54).

1.3.2. Salmochelin

Salmocheline sind chemisch durch Glykosylierung modifizierte Varianten des Enterobactins und zählen ebenfalls zu den Katecholaten (46, 55-57). Erstmals in *Salmonella* spp. beschrieben, konnte es unter den *Enterobacteriaceae* auch in *E. coli*, *Klebsiella pneumoniae* und *Shigella dysenteriae* identifiziert werden (46, 58). Auf dem sogenannten *IroA*-Lokus befinden sich die Gene *iroBCDE* und *iroN*, die in zwei konvergenten Operons organisiert sind (46, 56). Die C-Glykosyltransferase IroB ist in der Lage bis zu drei Glycosylgruppen an die 2,3-Dihydroxybenzoyl Einheiten des Enterobactins zu übertragen, wodurch mehrere zyklische und lineare Varianten der Salmocheline entstehen (55, 57, 59, 60). Durch die Modifizierung zu Salmochelinen entgehen pathogene Bakterien der Sequestrierung von Enterobactin durch das Lipocalin-2, was im Tiermodell das Virulenzpotential signifikant steigert (61). Insgesamt ist das Salmochelinsystem in mehreren Arbeiten als relevanter Virulenzfaktor für ExPEC bestätigt worden (62-65). Bemerkenswerterweise konnte für einzelne Elemente wie den Außenmembranrezeptor IroN zusätzliche Effekte unabhängig der Eisenaufnahme festgestellt werden, wie beispielsweise die Funktion als Internalisierungsfaktor für Urothelien (66).

1.3.3. Aerobactin

Unter den Siderophoren stellt Aerobactin ein Hydroxamat dar, das erstmals in *Aerobacter aerogenes* entdeckt wurde und in vielen pathogenen Isolaten in *E. coli*, *Shigella flexneri* und *Klebsiella pneumoniae* beschrieben wurde (67, 68). Die Dissoziationskonstante K_d mit $10^{-22,5}$ M bei physiologischem pH liegt damit im äquivalenten Bereich von eisenbeladenem Transferrin und deutlich unter den Werten ermittelt für Enterobactin und seinen Derivaten (67, 69, 70). Allerdings zeigt es als Hydroxamat eine gewisse Säurestabilität, die unter sauren Bedingungen eine höhere Bindungsaffinität für Eisen bedingt als Enterobactin (47). Dies ist ein Hinweis darauf, dass eine Selektion von mehreren Eisenaufnahmesystemen mit unterschiedlichen Funktionsoptima unter variablen Bedingungen stets eine adäquate Zufuhr von Eisen gewährleisten kann. Genetisch besteht das Aerobactin-Gencluster aus 5 Genen, den Genen *iucABCD* für Biosynthese und *iutA* kodiert für den spezifischen Rezeptor (46, 71). Es ist häufig auf Virulenz-Plasmiden lokalisiert, wurde aber auch chromosomal

in einigen Isolaten gefunden (72). Aerobactin wurde als wichtiger Virulenz- und Fitnessfaktor in Infektionsmodellen für ExPEC und vogelpathogene *E. coli*, sogenannte *avian pathogenic E. coli* (APEC), beschrieben (73-75).

1.3.4. Yersiniabactin

Anhand seiner chemischen Grundstruktur wird das Siderophor Yersiniabactin als Phenolat klassifiziert und wurde zuerst in *Yersinia enterocolitica* identifiziert (76). Zunächst in virulenten *Yersinia* spp. beschrieben, konnte das Siderophor-System verbreitet über weitere *Enterobacteriaceae* wie *E. coli*, *Pseudomonas* spp., *Klebsiella* spp. und *Citrobacter* spp. verifiziert werden (77-80). Mit einer Dissoziationskonstante K_d von $4x10^{-36}$ M zeigt Yersiniabactin eine sehr hohe Bindungsaffinität zu dreiwertigem Eisen und scheint für neutrale bis leicht alkalische pH Bedingungen effektiver zu sein (47, 81). Das Yersiniabactin-Gencluster besteht aus 11 Genen, die in 4 Operons organisiert sind, und auf einer genomischen Insel lokalisiert ist und übertragen werden kann, die aufgrund ihrer essentiellen Rolle für hochvirulente *Yersinia* spp. auch *High-Pathogenicity Island* (HPI) bezeichnet wird (48, 77). Experimentelle Ergebnisse für unterschiedliche Isolate in diversen Infektionsmodellen konnten den Stellenwert der HPI als relevanten Virulenzfaktor in ExPEC belegen (75, 82-85). Weitere Arbeiten unterstreichen die Multifunktionalität dieses Siderophores auf mehreren Ebenen. Yersiniabactin kann durchaus als universelles Metallophor angesehen werden, da es neben Eisen auch eine Affinität für Kupfer und Zink aufweist (86, 87). Es wirkt protektiv hinsichtlich Kupfertoxizität und Redox-basierten Phagozytose-Mechanismen und umgeht darüber hinaus der immunologischen Eisensequestrierung durch Verbindungen wie dem Lipocalin-2 im Rahmen von Infektionsprozessen (88, 89). Einzelne Elemente wie der spezifische Außenmembranrezeptor FyuA optimiert die Biofilmbildung in ExPEC und scheint sich als Vakzine-Kandidat besonders zu eignen (85, 90, 91). Eine komplexe Interaktion der HPI mit dem *E. coli* Metabolom wurde zudem berichtet (92-94).

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3. Fragestellungen

Das erste Projekt befasst sich mit der Multifunktionalität von Eisenaufnahmesystemen in ExPEC. Siderophore stellen relevante Virulenz– und Fitnessfaktoren für die Interaktion der pathogenen Erreger mit dem Wirt während einer Infektion dar. Dabei beschränkt sich die Hauptaufgabe nicht ausschließlich auf die Eisenaufnahme, sondern im Rahmen der komplexen Koordination von Pathomechanismen scheinen Siderophore und einzelne Elemente dieser Systeme eine Optimierung von diversen Prozessen zu gewährleisten. Ziel war es nun, den Beitrag einzelner Siderophor-Systeme während der Biofilmbildung in ExPEC zu untersuchen.

Ein Repertoire an bestimmten Virulenz – und Fitnessfaktoren ist die Voraussetzung, um sich optimal an eine spezielle Nische zu adaptieren. Die Koordination der Pathomechanismen spielt dabei eine entscheidende Rolle. Obwohl in den meisten Fällen alle notwendigen Enzyme für die Biosynthese eines bioaktiven Metaboliten auf definierten Genclustern für den entsprechenden Virulenzfaktor kodiert sind, teilen sich viele Synthesewege gemeinsame Schnittstellen. In virulenten ExPEC Isolaten der phylogenetischen Gruppe B2 besteht eine starke Assoziation der HPI mit einer Pathogenitätsinsel in unmittelbarer Nachbarschaft bezeichnet als die *pks* Insel, die für das Genotoxin Colibactin kodiert. Colibactin wird chemisch den Polyketiden zugeordnet und bewirkt DNA-Doppelstrangbrüche, Zellzyklusarrest in der G2 Phase und Megalozytose in infizierten eukaryotischen Zellen. Sowohl für Yersiniabactin als auch für Colibactin ist für die Biosynthese die Aktivität von Phosphopantetheinyl-Transferasen (PPTase) notwendig. In einem internationalen Kooperationsprojekt wurde die Interaktion beider Synthesewege mit Fokus auf PPTasen untersucht.

4. Zusammenfassung der publizierten Ergebnisse

Der Salmochelin-Rezeptor IroN selbst und nicht die Salmochelin-vermittelte Eisenaufnahme verbessert die Fähigkeit zur Biofilmbildung in ExPEC.

Giuseppe Magistro, Christiane Hoffmann, Sören Schubert

Int J Med Microbiol. 2015 Jun-Aug;305(4-5):435-45

Der Schlüssel zum Erfolg der EXPEC um Nischen außerhalb des Gastrointestinaltraktes zu besiedeln und Infektionen zu verursachen besteht im koordinierten Einsatz von Virulenz– und Fitnessfaktoren. Forschungsergebnisse konnten nicht nur ein besonderes Spektrum an Virulenzfaktoren mit spezifischer Funktion identifizieren, sondern deckten zudem den vielseitigen Einsatz dieser Faktoren auf. Insbesondere die Eisenaufnahmesysteme der ExPEC spielen in diesem Kontext eine besondere Rolle. Neben der Akquirierung von Eisen sind diese in der Lage bestimmte Virulenzeigenschaften zu optimieren. Im Rahmen der hier vorliegenden Arbeit wurde der Beitrag des Salmochelin-Siderophor-Systems zur Biofilmbildung der ExPEC untersucht. Unter den gleichen eisenlimitierten Bedingungen, wurde ein differenziertes Ansprechen der diversen Siderophore zunächst auf Transkriptionsebene festgestellt. Der spezifische Salmochelin-Rezeptor IroN zeigte eine starke Expression während der Biofilmbildung, wohingegen im gleichen Medium unter planktonischer Kultivierung keine Induktion beobachtet wurde. Eine Rezeptor Knockout-Mutante für *iroN* resultierte in einer reduzierten Biofilmproduktion um etwa 50 %. Die effektive Biofilmbildung wurde nicht durch gezielte Mutation des Syntheseapparates für Salmochelin beeinträchtigt. Somit erscheint der Effekt des Rezeptors IroN für die Biofilmbildung unabhängig von der Eisenaufnahme durch das Siderophor selbst bedingt zu sein. Die verstärkte Expression des Rezeptors erbrachte keine signifikante Verbesserung für die Biofilmbildung in ExPEC. Interessanterweise, konnte die Fähigkeit der Biofilmformation in einem schwachen Biofilmbildner wie einem apathogenen *E. coli* K12 Stamm weder durch zusätzliche Expression des Rezeptors IroN noch durch Ausstattung mit einem kompletten funktionalen Salmochelin-System verbessert werden. Jedoch zeigte in einem ExPEC Stamm die Restitution nur des Rezeptors

IroN in einer Deletionsmutante für das komplette Salmochelin-Gencluster die vollständige Wiederherstellung der Kapazität zur Biofilmformation wie im Wildtypstamm. Dies bedeutet, dass der Effekt des Aussenmembranrezeptors IroN für die Fähigkeit zur Biofilmbildung einen bestimmten genetischen Hintergrund voraussetzt, der in ExPEC gewährleistet ist, nicht jedoch in apathogenen *E. coli* K12 Stämmen. Durch die hier präsentierten Resultate konnte der wesentliche Beitrag des Salmochelinrezeptors IroN für die Biofilmbildung aufgezeigt werden und somit ein weiterer Beleg für die vielseitige Anwendung der Eisenaufnahmesysteme in ExPEC erbracht werden.

Beitrag der Autoren:

Giuseppe Magistro und Sören Schubert konzipierten gemeinsam das Projekt. Giuseppe Magistro übernahm die Projektplanung und führte alle Experimente eigenverantwortlich durch. Die Datenauswertung und Interpretation der Ergebnisse wurde gemeinsam von Giuseppe Magistro, Christiane Hoffmann und Sören Schubert übernommen. Giuseppe Magistro und Sören Schubert waren für die Fertigstellung des Manuskriptes verantwortlich.

Die Interaktion zwischen Siderophoren und dem Genotoxin Colibactin Biosynthesewegen in *Escherichia coli*

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PLoS Pathog. 2013;9(7)

In *Escherichia coli* benötigen die Biosynthesewege diverser niedermolekularer Verbindungen zur Eisenaufnahme, bekannt als Siderophore, und des Genotoxins Colibactin das Enzym 4'-Phosphopantetheniyltransferasen (PPTase). Bisher wurden nur zwei solcher PPTasen identifiziert: EntD und ClbA. Das Gen für das Enzym EntD (*entD*) ist auf dem *E. coli* Kerngenom lokalisiert, während sich *clbA* auf der *pks* Pathogenitätsinsel, die für Colibactin kodiert, befindet. Interessanterweise ist die *pks* Insel genetisch streng mit der *High Pathogenicity Island* (HPI) in einer Untergruppe hochvirulenter *E. coli* Stämme assoziiert. Die HPI beinhaltet alle notwendigen Gene für die Yersiniabactin-Synthese außer einem Gen für die HPI-spezifische PPTase. In der vorliegenden Arbeit wurde die mögliche Interaktion der Synthesewege für Siderophore (u.a. Yersinabaktin) und Colibactin durch den funktionellen Austausch der PPTasen EntD und ClbA untersucht. Wir konnten demonstrieren, dass ClbA die Siderophorproduktion aufrechterhalten kann. Die Inaktivierung beider Gene *entD* und *clbA* hatte einen dramatischen Effekt auf die Virulenz extraintestinal pathogener *E. coli* (ExPEC) im murinen Sepsismodel. Die Aktivität eines der Enzyme EntD oder ClbA war für das Überleben der ExPEC *in vivo* notwendig. Dies ist der erste Beweis für die Interaktion diverser PPTase-abhängiger Biosynthesewege für funktionell unterschiedliche sekundäre Metabolite in einem Mikroorganismus. Daher liegt der Schluss nahe, dass die strikte genetische Assoziation der *pks* Insel und der HPI in hochvirulenten *E. coli* selektioniert wurde, weil es sich bei ClbA um eine promiskuitive PPTase handelt, die sowohl zur Synthese von Colibactin als von Siderophoren beiträgt. Die vorliegenden Ergebnisse heben die komplexe regulatorische Interaktion diverser Virulenzfaktoren hervor. Die Identifizierung gemeinsamer Schnittstellen erlaubt nicht nur Einblick in die Virluenzmechanismen der ExPEC zu gewinnen, sondern auch vielversprechende, therapeutische Zielstrukturen für die Entwicklung sogenannter Anti-Virulenzstrategien zu entwickeln.

Beitrag der Autoren:

Patricia Martin, Ingrid Marcq, Jean-Philippe Nougayrède und Eric Oswald konzipierten das Projekt und planten die Experimente. Patricia Martin, Ingrid Marcq, **Giuseppe Magistro**, Marie Penary, Christophe Garcie, Delphine Payros, Michèle Boury, und Maiwenn Olier führten die Experimente durch. Patricia Martin, **Giuseppe Magistro**, Maiwenn Olier, Marc Audebert, Jean-Philippe Nougayrède, Sören Schubert und Eric Oswald analysierten die Daten. Marc Audebert und Christian Chalut stellten Reagenzien, Materialien und Analyseinstrumente zur Verfügung. Patricia Martin und Eric Oswald verfassten das Manuskript.

5. Summary of published data

The salmochelin receptor IroN itself, but not salmochelin-mediated iron uptake promotes biofilm formation in extraintestinal pathogenic *Escherichia coli* (ExPEC)

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Int J Med Microbiol. 2015 Jun-Aug;305(4-5):435-45

The key to success of extraintestinal pathogenic *Escherichia coli* (ExPEC) to colonize niches outside the intestinal tract and to establish infection is the coordinated action of numerous virulence and fitness factors. Intense research revealed not only an arsenal of unique virulence determinants with specific action, but also the multi-functionality of single elements. Especially iron uptake systems of ExPEC proved to be of prime importance. Apart from iron acquisition they optimize certain virulence properties. Here we analyzed the contribution of the salmochelin siderophore system to the ability of ExPEC to form biofilms. In the same iron-limited environment, ExPEC displayed a distinct transcriptional profile of siderophore systems. During biofilm formation the *iroN* gene coding for the specific receptors of the siderophore salmochelin was highly up-regulated. Almost no induction was observed during planctonic growth. Disruption of *iroN* resulted in a reduction of almost 50 % in biofilm production. Efficient biofilm formation was not affected in a salmochelin synthesis mutant. Thus, the contribution of IroN is independent from the ability to produce salmochelin. Enhanced expression of IroN did not increase significantly the capacity to form biofilms in ExPEC. Interestingly, the additional expression of IroN or even the acquisition of the entire salmochelin system was not able to improve biofilm formation in a poor biofilm producer like a laboratory *E. coli* K12 strain. However, complementation with only *iroN* in an ExPEC *iroA* deletion mutant was able to restore biofilm formation. The contribution of IroN to biofilm formation appears to require a certain background found in ExPEC, but not in *E. coli* K12. This study identified the contribution of IroN to biofilm formation and highlights the multi-functional role of iron uptake systems in ExPEC.

Author contributions:

Giuseppe Magistro and Sören Schubert conceived the project. Giuseppe Magistro designed and performed the experiments. Giuseppe Magistro, Christiane Hoffmann and Sören Schubert analyzed and interpreted the data. Giuseppe Magistro and Sören Schubert wrote the manuscript.

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

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PLoS Pathog. 2013;9(7)

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 (*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 interchangeability 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 phosphopantetheinyl-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.

Author contributions:

Conceived and designed the experiments: Patricia Martin, Ingrid Marcq, Jean-Philippe Nougayrède, Eric Oswald. Performed the experiments: Patricia Martin, Ingrid Marcq, **Giuseppe Magistro**, Marie Penary, Christophe Garcie, Delphine Payros, Michèle Boury, Maiwenn Olier. Analyzed the data: Patricia Martin, **Giuseppe Magistro**, Maiwenn Olier, Marc Audebert, Jean-Philippe Nougayrède, Sören Schubert, Eric Oswald. Contributed reagents/materials/analysis tools: Marc Audebert, Christian Chalut. Wrote the paper: Patricia Martin, Eric Oswald.

6. Veröffentlichungen

6.1. Veröffentlichung I (Erstautorenschaft)

International Journal of Medical Microbiology 305 (2015) 435–445

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

The salmochelin receptor IroN itself, but not salmochelin-mediated iron uptake promotes biofilm formation in extraintestinal pathogenic *Escherichia coli* (ExPEC)

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ARTICLE INFO

Article history:
Received 18 August 2014
Received in revised form 22 March 2015
Accepted 24 March 2015

Keywords:
Siderophores
IroN
Biofilm
Extraintestinal pathogenic *Escherichia coli*
Urinary tract infection

ABSTRACT

The key to success of extraintestinal pathogenic *Escherichia coli* (ExPEC) to colonize niches outside the intestinal tract and to establish infection is the coordinated action of numerous virulence and fitness factors. Intense research revealed not only an arsenal of unique virulence determinants with specific action, but also the multi-functionality of single elements. Especially iron uptake systems of ExPEC proved to be of prime importance. Apart from iron acquisition they optimize certain virulence properties. Here we analyzed the contribution of the salmochelin siderophore system to the ability of ExPEC to form biofilms. In the same iron limited environment, ExPEC displayed a distinct transcriptional profile of siderophore systems. During biofilm formation the *iroN* gene coding for the specific receptors of the siderophore salmochelin was highly upregulated. Almost no induction was observed during planctonic growth. Disruption of *iroN* resulted in a reduction of almost 50% in biofilm production. Efficient biofilm formation was not affected in a salmochelin synthesis mutant. Thus, the contribution of IroN is independent from the ability to produce salmochelin. Enhanced expression of IroN did not increase significantly the capacity to form biofilms in ExPEC. Interestingly, the additional expression of IroN or even the acquisition of the entire salmochelin system was not able to improve biofilm formation in a poor biofilm producer like a laboratory *E. coli* K12 strain. However, complementation with only IroN in an ExPEC *iroA* deletion mutant was able to restore biofilm formation. The contribution of IroN to biofilm formation appears to require a certain background found in ExPEC, but not in *E. coli* K12. This study identified the contribution of IroN to biofilm formation and highlights the multi-functional role of iron uptake systems in ExPEC.

Introduction

Urinary tract infections (UTI) are among the most common bacterial infections. With its contribution to infectious morbidity, mortality and financial costs to healthcare systems worldwide, UTIs emerge more and more as a public health issue of substantial socioeconomic importance. In the United States, estimated 6–8 million cases of uncomplicated cystitis were responsible for health care costs of approximately 1.6 billion dollars (Russo and Johnson, 2006). It is estimated that almost 50% of women will at least experience one UTI during their lifetime, with 25% of these women likely to develop recurrence within the next 6–12 months (Foxman et al., 2000; Foxman, 1990). The primary etiological pathogens of UTI are extraintestinal pathogenic *E. coli* (ExPEC). Among ExPEC, uropathogenic *E. coli* (UPEC) account for approximately 90% of all UTIs among ambulatory populations (Marrs et al., 2005) and are isolated in 50% of nosocomial UTIs (Kucheria et al., 2005). These UPEC reside in the intestinal tract as part of the normal intestinal flora, but as soon as they reach extraintestinal niches as the urinary tract, the orchestrated action of various virulence factors (VF) aids in efficient colonization and establishment of infection (Kohler and Dobrindt, 2011; Johnson and Russo, 2002; Nielubowicz and Mobley, 2010). In the past, the management of UTI relying on the use of antibiotics was quite efficient due to the high antimicrobial susceptibility of UPEC. Unfortunately, the increasing numbers of antimicrobial resistances will render the future therapy more challenging and more expensive than ever. New preventive and therapeutic approaches are needed to treat UTI in the near future. Deciphering the molecular mechanisms underlying the pathogenesis of UTI by UPEC may reveal promising targets in order to develop potent alternatives to antibiotics. Scientific findings accumulated over the last decades

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http://dx.doi.org/10.1016/j.ijmm.2015.03.008
1438-4221/

have helped to elucidate the pathogenic potential of extraintestinal pathogenic *E. coli* (ExPEC) (Johnson and Russo, 2002; Johnson, 1991; Kaper et al., 2004). It is striking to note that iron acquisition systems proved to be of paramount importance for full virulence of ExPEC. To face the iron scarcity of the urinary tract, ExPEC have evolved a multifactorial strategy to scavenge efficiently for this nutrient (Garenaux et al., 2011). Different low-molecular weight compounds termed siderophores are secreted to bind ferric iron with high affinity from the host. While laboratory *E. coli* K12 strains are able to produce only the catecholate siderophore enterobactin, ExPEC produce a variety of different siderophores with specific chemical properties. The repertoire comprises the catecholates enterobactin and salmochelin, the hydroxamate aerobactin and the mixed-type siderophore yersiniabactin. Specific outer membrane receptors are expressed to optimize siderophore-mediated iron uptake. Of note, an additional array of siderophore receptors is expressed to utilize alternative siderophores produced by different microorganisms. The iron regulated outer-membrane receptors FhuA is expressed to take up hydroxamate siderophores like ferrichrome, originally secreted by soil fungi (Garenaux et al., 2011). The salmochelins represent C-glycosylated derivatives of enterobactin and 2,3-dihydroxybenzoyl serine molecules (DHBS) (Bister et al., 2004; Fischbach et al., 2005; Hantke et al., 2003; Lin et al., 2005; Zhu et al., 2005). The *iroA* locus contains two convergent operons, namely *iroN* encoding the specific salmochelin receptor IroN and *iroBCDE* coding for genes required for synthesis, secretion and transport (Hantke et al., 2003; Sorsa et al., 2003). Interestingly, certain siderophores can be bound with lower affinity by alternative outer membrane receptors. So salmochelins are not exclusively bound by IroN, but also by FepA, the receptor of enterobactin, and the iron regulated outer membrane protein Cir (Hantke et al., 2003). FepA and Cir are expressed both by ExPEC and laboratory *E. coli* K12. Apart from iron acquisition elements of these iron uptake systems turned out to be multi-functional. For example, IroN additionally influences the invasive properties of ExPEC (Feldmann et al., 2007). Due to the high prevalence among ExPEC and its essential role to virulence (Negre et al., 2004; Russo et al., 2002), this outer membrane protein has been evaluated as target for vaccine development. Indeed, being pathogen-specific, antigenic, surface exposed and *in vivo* expressed, iron regulated outer membrane proteins fulfil all essential criteria of a potential vaccine candidate. Interestingly, IroN proved to be a promising candidate in vaccinology in many studies (Wieser et al., 2010, 2012; Alteri et al., 2009; Durant et al., 2007; Brumbaugh et al., 2013). Moreover, a study by Hancock et al. reported that outer membrane receptors may be involved in efficient biofilm formation in human urine (Hancock et al., 2008). The ability of UPEC to form biofilm plays a role for the colonization of indwelling medical devices such as catheters. It received even more attention in recent years as a growing body of evidence supports the concept, that biofilm like communities of UPEC within the host cell termed intracellular bacterial communities (IBCs) may be responsible for recurrent and chronic infection (Anderson et al., 2003). The main objective of the current study is to investigate the contribution of the salmochelin receptor IroN to biofilm formation.

Materials and methods

Bacterial strains and media

Bacterial strains and plasmids used in the present work are listed in Table 1. The archetypal UPEC strains NU14 and 536 were isolated from patients suffering from cystitis and pyelonephritis, respectively (Hultgren et al., 1986; Berger et al., 1982). Bacteria were cultured in Luria-Bertani (LB) medium and artificial urine medium (AUM) (Brooks and Keevil, 1997). We decided to perform the experiments in AUM instead of native human urine in order to obtain results under absolute standardized conditions. Siderophore production was determined by spotting standardized cultures on CAS agar plates (Schwyn and Neilands, 1987). Use of antibiotics was provided as necessary (chloramphenicol 20 μg/ml, kanamycin 25 μg/ml, ampicillin 100 μg/ml, tetracycline 12 μg/ml). Growth curves in AUM were established in triplicates of 50 ml cultures in 250 ml Erlenmeyer flasks. Overnight cultures were incubated in 50 ml fresh AUM to a starting OD_{600nm} of 0.05 and optical density at 600 nm was recorded every 60 min. Final cell density was determined at $OD_{600nm} = 0.3$.

Biofilm formation assays

Biofilm assays were performed in AUM according to O'Toole and Kolter (1998). For this, 96 well plates were used for the

Table 1
Strains and plasmids.

Strains	Relevant characteristics	References
NU14 wt	O18: K1: H7; cystitis isolate	Hultgren et al. (1986)
NU14 *fyuA::Cm*	Isogenic *fyuA* mutant	This study
NU14 *iroN::Kn*	Isogenic *iroN* mutant	This study
NU14 *iroA::Kn*	Deletion of the entire *iroA* locus	This study
NU14 *iroA::Kn* + *IroN*	*iroA* locus deleted, expressing IroN (pACYC184-*iroN*)	This study
NU14 *iroA::Kn* vc	*iroA* locus deleted, vector control (pACYC184)	This study
NU14 *iroN::Kn* rec high	Recomplemented *iroN* mutant, pUC19-*iroN*	This study
NU14 *iroN::Kn* rec low	Recomplemented *iroN* mutant, pACYC184-*iroN*	This study
536 wt	O6: K15: H31; pyelonephritis isolate	Berger et al. (1982)
MG1655	λ -, ilvG, rfb-50 rph-1	Blattner et al. (1997)
MG1655 vc	Vector control, pACYC184	This study
MG1655 + FyuA	Expressing FyuA, pACYC184-*fyuA*	This study
MG1655 + IroN	Expressing iroN, pACYC184-*iroN*	This study
MG1655 + iro	Produces salmochelin, p332	This study
Plasmids		
pKD3	Chloramphenicol template plasmid	Datsenko and Wanner (2000)
pKD4	Kanamycin template plasmid	Datsenko and Wanner (2000)
pKD46	Lambda red recombinase helper plasmid	Datsenko and Wanner (2000)
pACYC184	Medium-copy plasmid, Cm	Chang and Cohen (1978)
pUC19	High-copy plasmid, Ap	Yanisch-Perron et al. (1985)
p332	Cosmid carrying *iroA* locus (*iroBCDEN*), Ap, Kn	Sorsa et al. (2003)
pACYC184-*fyuA*	Expressing FyuA, Cm	This study
pACYC184-*iroN*	Expressing IroN, Cm	This study
pUC19-*iroN*	Expressing IroN, Ap	This study

Table 2
Primers.

Genes	Primers	Sequences (5′–3′)
Primers for gene disruption		
fyuA	*fyuA*.KO.for	GGCGACATGATTAACCCCGCGACGGGAAGCGATGACTTAGGTGTAGGCTGGAGCTGCTTC
	fyuA.KO.rev	CGATATCAAAACGATCGGTTAAATGCCAGGTCAGGTCACTCATATGAATATCCTCCTTA
iroN	*iroN*.KO.for	ATACCCGCGGTGACACCAACTGGGTGCCACCGGAACAGGTTGAGCGTATTGAAGTGATGTGTAGGCTGGAGCTGCTTC
	iroN.KO.rev	TCCCCACCAGTGAATAAGCGCCCAGCTCTTTACCTGACAGACCGCCAGTATCTTCCATATGAATATCCTCCTTA
iroB	*iroB*.KO.for	GTGCGTCGACTGCCTGATTTAGATCGTCAAGCGGAGAGGGGTGTAGGCTGGAGCTGCTTC
	iroB.KO.for	CTACCCTTTCTGTACCATTGTGATCAGGCTTTTCGCCACTCATATGAATATCCTCCTTAG
iroA	*iroA*.KO.for	ATGAGAATTAACAAAATCCTCTGGTCGCTAACTGTGCTCCTGGTTGGGTTGTGTAGGCTGGAGCTGCTTC
	iroA.KO.rev	ATGCGTATTTTGTTTGTAGGCCCGCCTCTCTATGGGCTGTTGTATCCTGTCATATGAATATCCTCCTTAG
Primers and probes for TaqMan-PCR		
16S rDNA	*16S rDNA*.for	TTGACGTTACCCGCAGAAGAA
	16S rDNA.rev	GCTTGCACCCTCCGTATTACC
	16S rDNA.probe	CGGCTAACTCCGTGCCAGCAGC
fyuA	*fyuA*.for	ACACCCGCGAGAAGTTAAATTC
	fyuA.rev	AGCGGTGGTATAGCCGGTACT
	fyuA.probe	CCTACGACATGCCGACAATGCCTTATTTAA
iroN	*iroN*.for	GCTGACCGTTGGTGCAGAGT
	iroN.rev	CATTCACCGTCAGGCTGGTA
	iroN.probe	CCGCGATAAGCTCGATGATCCTTCC
fepA	*fepA*.for	AATTCAGCTTCCGCTTGTATGG
	fepA.rev	ACTGATGGCCCTGGTTGATATC
	fepA.probe	ACAAAACCCAGGCTGACGCGTG
chuA	*chuA*.for	ACCATTTGTCGGCATCAACAT
	chuA.rev	TTGGCGGAACCCGCTAT
	chuA.probe	AGCCGTCGCTGCTACCGCGA
cir	*cir*.for	TTACCAGCGGACCATTAATTGA
	cir.rev	GGGTCATCCTTTTCACGTTTTG
	cir.probe	CTGGGAATGAAAGCTTACGGCAGCCT
fhuA	*fhuA*.for	GGCCGGCTGCAACTATTG
	fhuA.rev	TGAATCGGCGTATCGGTTTT
	fhuA.probe	CGCGACAGTCAGCTACCGGCA

quantification of biofilm production and 6 well flat-bottom plates were used to prepare samples for transcriptional (real-time PCR) and translational (Western blotting) analysis. Strains were incubated statically for 24 h at 37 °C in AUM. After inversion of the plates to remove unbound bacteria, adherent UPEC were stained with 0.1% of crystal violet for 15 min. Excess dye was removed followed by three washing steps using PBS buffer. Then crystal violet was solubilized with ethanol/acetone solution (80:20, v/v) and measured in a plate reader at OD_{590nm}. In order to investigate the impact of different concentrations of ferric iron on biofilm forming capacity, AUM was supplemented with $FeCl_3$ (1 μM, 10 μM, 100 μM). Each strain tested was grown in three wells and experiments were repeated at least three times. In order to gain sufficient material for RNA extraction or western blotting, cells were incubated in three wells of a 6 well plate.

Construction of isogenic mutants

Deletion mutants of target genes were generated using the lambda red recombinase approach by Datsenko and Wanner (2000). Briefly, primers with 40-nt homology extensions to the 5′- and 3′-regions of the gene of interest and 20-nt priming sequences for the template plasmids pKD3 or pKD4 carrying resistance cassettes flanked by FRT recognition target sites were designed (Table 2). The resulting PCR product was then transformed into strains harboring the helper plasmid pKD46 with lambda red recombinase under control of an arabinose-inducible promoter. In case of successful replacement of the specific gene, Kn^R- or Cm^R-transformants were selected. Correct integration of the resistance cassette was confirmed by PCR.

Cloning and recombinant DNA techniques

Standard genetic methods were performed mainly as described by Sambrook and Russell (2001). Enzymes were purchased from Fermentas (Schwerte, Germany) and used according to the manufacturer's recommendations. Primers and plasmids used in this study are listed in Tables 1 and 2. Plasmids for complementation of the deleted genes were constructed by PCR amplification of the wild-type alleles under the control of their native promoter. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and cloned into medium-copy plasmid pACYC184 (Chang and Cohen, 1978) or high-copy plasmid pUC19 (Yanisch-Perron et al., 1985) *via* suitable restriction sites.

Western blotting

For detection of FyuA and IroN expression, bacteria were cultivated as described above. After incubation in AUM for 24 h at 37 °C unbound cells were removed by inversion and plates were washed three times with PBS. Strains were scraped off thoroughly, resuspended in PBS and finally adjusted to an OD_{600} = 1.0. Bacteria from this standardized culture were pelleted by centrifugation and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subsequently transferred to a Protran® nitrocellulose transfer membrane. Rabbit polyclonal antiserum to FyuA and IroN (Feldmann et al., 2007) were used as primary antibody.

RNA extraction and quantitative real-time PCR (TaqMan)

Different RNA samples were prepared as described above. RNA extraction was performed using the Trizol (Invitrogen, Darmstadt, Germany) method (Chomczynski and Sacchi, 1987). Total RNA was first treated with DNase I (Fermentas) to remove contaminating genomic DNA. After that, first-strand cDNA was synthesized using random hexamers and RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) according to the manufacturer's recommendation. TaqMan-PCR was run on a 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). Primers were

designed using the Primer Express software (Version 3.0, Applied Biosystems) and probes were labeled with FAM at the 5′-terminus and TAMRA at the 3′-terminus (Table 2). TaqMan-PCR reactions were carried out in a final volume of 25 μl containing TaqMan Gene Expression Master Mix (Applied Biosystems, Darmstadt, Germany), primers, probe and 30 ng of cDNA. The transcript levels were normalized to that of 16S rDNA. Data were analyzed by the $2^{-\Delta\Delta C_T}$ method as described by Livak and Schmittgen (2001). Growth in LB medium served as calibrator because iron scarcity can be excluded in this iron rich medium.

Results

Transcriptional and translational analysis of siderophore receptors during biofilm formation

The first step to study the role of outer membrane siderophore receptors for biofilm formation was the transcriptional analysis of relevant genes by real-time PCR. For this, a selection of genes coding for siderophore receptors, namely *fyuA*, *fhuA*, *fepA*, *iroN*, and *cir*, was made. We also included *chuA*, which encodes a receptor for heme uptake. The transcriptional profile of UPEC strains 536 and NU14 during planctonic growth in artificial urine medium (AUM) was compared with bacteria during biofilm formation in AUM. As depicted in Fig. 1, both UPEC strains showed a similar transcriptional profile, with *fepA*, *chuA*, and *cir* presenting the most upregulated genes during planctonic growth in AUM. Interestingly, the profiles changed considerably during biofilm formation with *fyuA* and *iroN* being strongly upregulated. In strain 536 the transcription of *fyuA* and *iroN* increased 28- and 11-fold (each $P < 0.001$), respectively. Isolate NU14 demonstrated also a 20- and 18-fold upregulation of *fyuA* and *iroN* genes (each $P < 0.001$), respectively. The transcription of the *fepA* gene was slightly increased in strain 536, whereas in *E. coli* strain NU14 a fourfold induction of *fepA* gene expression could be observed ($P < 0.001$). Transcription of *chuA* and *cir* decreased significantly in both UPEC strains (each $P < 0.001$). The *fhuA* gene coding for a hydroxamate receptor was slightly upregulated during biofilm formation in both strains. This transcriptional analysis revealed that the process of biofilm formation induced a significant upregulation of transcription of *fyuA* and *iroN* in both UPEC isolates. Next, we investigated whether the results obtained on the transcriptional level could be confirmed on the translational level. So we performed western blotting with standardized samples to compare the expression of FyuA and IroN during planctonic growth and biofilm formation in AUM (Fig. 2). In agreement with our observations obtained in the transcription assays, planctonic growth in AUM did not induce significantly the protein expression of FyuA and IroN in the two UPEC strains. The overall expression of FyuA and IroN was rather low during planctonic growth. This changed when bacteria were harvested from biofilm produced in AUM. Protein expression was considerably stronger for both FyuA and IroN. This data suggests that both siderophore receptors FyuA and IroN are relevant for efficient biofilm formation in UPEC. We focused on the relevance of IroN for the biofilm forming capability of UPEC, which has not been investigated so far. The following experiments were performed with isolate NU14.

Deletion of iroN *reduces biofilm formation*

We generated an isogenic *iroN* mutant of *E. coli* strain NU14 to investigate the role of IroN for biofilm formation. Quantification of biofilm production was tested in microtiter plates incubated statically in AUM for 24 h at 37 °C. The knockout strain showed a significant reduction in biofilm formation (49%; $P < 0.001$) compared to the wildtype strain, whereas the synthesis mutant NU14 Δ*iroB* was not affected ($P > 0.05$). Complete deletion of the *iroA* locus resulted in a significant impairment in biofilm production (42%; $P < 0.001$) (Fig. 3A). Complementation of IroN *in trans* was carried out to confirm that the phenotype was due to the deletion of *iroN* (Fig. 3B). We cloned *iroN* both into the medium-copy vector pACYC184 (Chang and Cohen, 1978) and the high-copy plasmid pUC19 (Yanisch-Perron et al., 1985). Expression levels were confirmed by western blotting (data not shown). In this experimental setting, we aimed to restore the ability of the knockout strain to produce efficiently biofilm like the wildtype strain. Moreover, by using high and medium copy expression we tried to determine, if an increased expression of IroN might influence the capacity to form biofilm. As shown in Fig. 3B, both complementation plasmids were able to restore biofilm production. However, we could not observe a significant difference between high- and medium-copy expression. These experiments confirmed that IroN contributes to efficient biofilm formation. Stronger expression of IroN did not enhance the biofilm forming capacity of the knockout strain. The deletion mutant NU14 Δ*iroA* lacks the entire salmochelin system. This mutant is not able to produce salmochelins or to take them up *via* the specific salmochelin receptor IroN. Interestingly, the exclusive expression of IroN in strain NU14 Δ*iroA* restored completely biofilm forming capacity. Disruption of the siderophore synthesis in the mutant NU14 Δ*iroB* did not interfere with the ability to form biofilm. Thus, the contribution of the salmochelin systems to biofilm formation requires the outer membrane receptor IroN and not the siderophore salmochelin. Growth experiments in AUM were carried out to evaluate whether the *iroN* effect on biofilm formation can be attributed to growth limitation of the mutant strain (Fig. 4A). No growth deficiency could be observed for the mutant strain NU14 Δ*iroN* compared to the wildtype strain. No significant difference for final cell density in AUM was observed between wildtype and knockout strain (Fig. 4B). The colony forming units per millilitre (cfu/ml) at $OD_{600nm} = 0.3$ for NU14 wildtype and NU14 Δ*iroN* were 4.0×10^8 ($\pm 1.5 \times 10^7$) and 3.9×10^8 ($\pm 1.5 \times 10^7$), respectively ($P > 0.05$).

Iron concentration modulates biofilm formation

Iron scarcity in AUM resulted in the upregulation of siderophore receptors, as demonstrated above. Iron as limiting factor was shown to modulate the capacity to form biofilms (Hancock et al., 2008; Rowe et al., 2010; Hancock et al., 2010; Alves et al., 2010). The influence of ferric iron supplementation on biofilm formation in AUM was investigated in the next experimental setting (Fig. 5). With the addition of rising concentration of $FeCl_3$ to the medium, biofilm production of the wildtype strain increased continuously. While 1 μm of ferric iron had no effect, 10 μM and 100 μM increased biofilm formation by 76.5% and 117.3%, respectively (each $P < 0.001$). An adequate response to iron supply with regard to biofilm formation could be observed for NU14 wildtype. In case of NU14 Δ*iroN*, 1 μM of ferric iron was not able to affect biofilm production neither. Upon supplementation with 10 μM $FeCl_3$, also the *iroN* mutant responded effectively in terms of biofilm formation. A significant increase of 123.9% ($P < 0.05$) was detected. This outcome indicates that the mutant strain was completely able to utilize the supplementation of ferric iron. Interestingly, no further promotion could be identified for 100 μM of ferric iron. The deficiency in biofilm formation of NU14 Δ*iroN* compared to NU14 wildtype was observed for any additional concentration of iron ($P < 0.001$). Although the *iroN* mutant was demonstrated to respond adequately to the addition of ferric iron, the absence of IroN impaired significantly biofilm formation compared to the wildtype. Not even abundance of ferric iron was able to compensate for the loss of the IroN outer membrane receptor relative to wildtype levels.

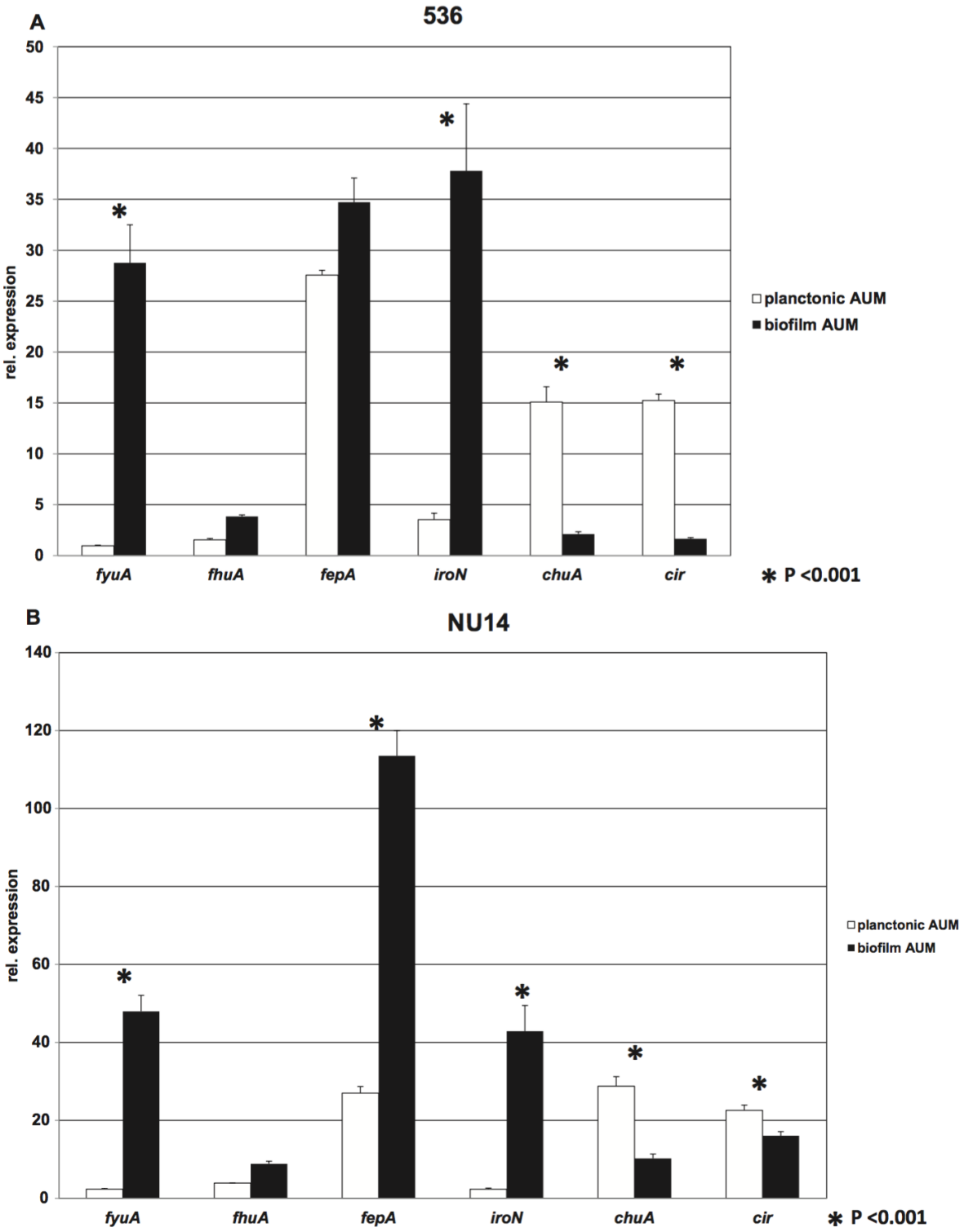

Fig. 1. Transcriptional analysis of UPEC during planctonic growth and in biofilms. Data were normalized to 16S rDNA. Strains grown in LB at 37 °C served as calibrator. Gene expression of strains during planctonic growth in AUM and during biofilm formation in AUM was compared. (A) UPEC strain 536. (B) UPEC strain NU14. The transcription rates of *fyuA* and *iroN* were significantly increased in biofilms relative to planctonic cultivation ($P < 0.001$).

Influence of IroN and the iroA *locus in laboratory* E. coli *K12 strain MG1655 on biofilm formation*

We demonstrated the relevance of IroN for efficient biofilm formation in UPEC. Next, we tried to understand whether IroN itself is able to improve biofilm formation or the entire *iroA* gene cluster (*iroBCDEN*) is necessary. This gene cluster is responsible for the production of the catecholate siderophore salmochelin and its receptor IroN. Therefore, we introduced either the *iroN* or the *iroA* locus into *E. coli* K12 strain MG1655 (Blattner et al., 1997). This strain only

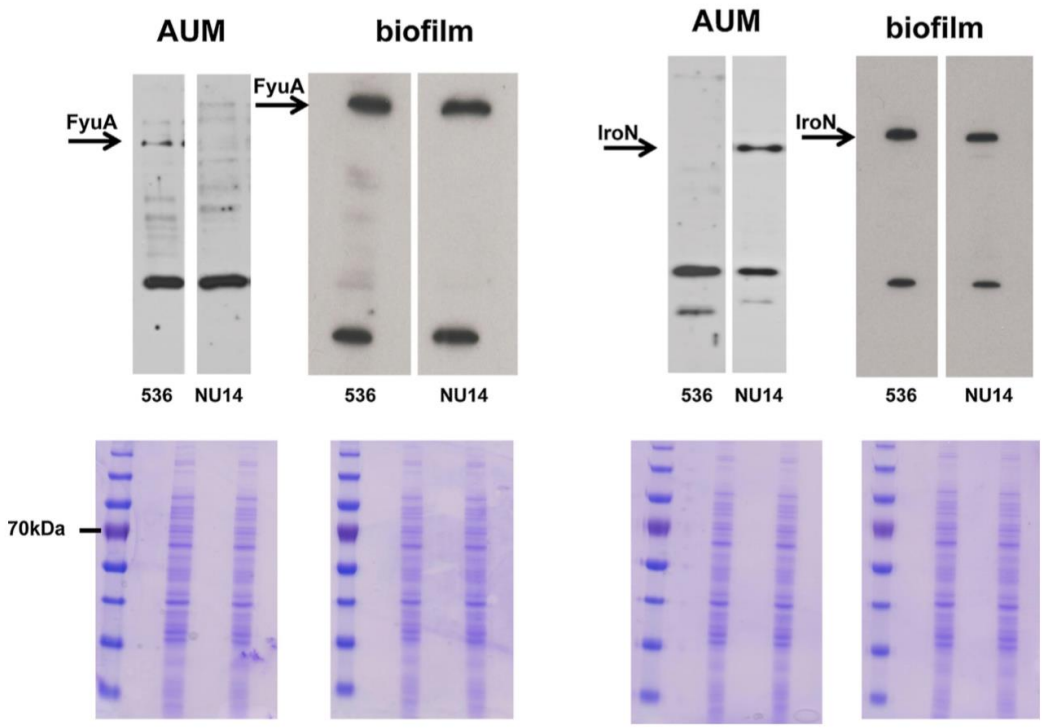

Fig. 2. Detection of FyuA and IroN during planctonic growth and in biofilms. Expression of siderophore receptors was investigated by immunoblotting. Almost no expression of FyuA was detected during planctonic cultivation, and IroN was only expressed in strain NU14. In contrast, during biofilm formation strong expression of both IroN and FyuA could be observed in strains 536 and NU14. SDS-Pages were performed as loading control of standardized samples.

produces the siderophore enterobactin, but is not able to express IroN or to synthesize salmochelin. The *E. coli* strain MG1655 is considered to be a poor biofilm producer. The additional expression of siderophore receptor IroN or FyuA did not result in stronger biofilm production in strain MG1655 (Fig. 6B). The *E. coli* K12 strain lacks most of the important iron uptake systems found in UPEC strains. So we transformed cosmid p332 (Sorsa et al., 2003), carrying the entire functional *iroA* locus, into *E. coli* MG1655. Additional siderophore secretion was confirmed on CAS agar plates (Fig. 6A). Standardized cultures were spotted on CAS agar plates and the diameter of the yellow halo around the center was determined. After 16 h of incubation, the halo of the wildtype strain was 0.9 cm (±0.1 cm). Additional siderophore production was observed in strain *E. coli* strain MG1655 + *iroA* with a diameter of 1.5 cm (±0.1 cm) ($P < 0.01$). Although an additional siderophore system was introduced in *E. coli* strain MG1655 + *iroA* no significant improvement in biofilm production could be detected (Fig. 6B). Thus, the expression of the single siderophore receptor IroN or the acquisition of the entire salmochelin system was not able to improve biofilm formation in an *E. coli* K12 background.

Discussion

The extraintestinal pathogenic *Escherichia coli* (ExPEC) are capable of colonizing niches outside the intestinal tract and to establish an infectious disease by means of the coordinated action of various virulence and fitness factors. Over the past years several reports have provided evidence for the essential role of iron uptake systems to the overall fitness. To face the iron scarcity of the urinary tract, ExPEC have evolved a strategy to scavenge efficiently for this nutrient. Although the supply of ferric iron to the microbial cell is considered to be the main function, recent studies indicate the implication of siderophore systems in various processes apart from just iron acquisition. The so called *iroA* gene cluster (*iroBCDEN*) is responsible for the production, secretion and uptake of the catecholate siderophore salmochelin (Muller et al., 2009). The outer membrane receptor IroN displays different functions. It obviously takes up salmochelin (Bister et al., 2004), but it can also contribute to invasion of urothelial cells (Feldmann et al., 2007). With IroN being pathogen-specific, antigenic, surface exposed and *in vivo* expressed it fulfils all essential criteria of a potential vaccine candidate. In the present study we aimed to analyze the contribution of siderophore receptors to biofilm formation. Planctonic cultivation in artificial urine medium resulted in increased transcription of genes coding for siderophore receptors due to iron limitation. The genes *fepA*, *chuA* and *cir* were the most upregulated ones, whereas transcription rates of the *fyuA* and *iroN* genes were not significantly increased. This first observation indicated that uptake of the respective siderophores yersiniabactin and salmochelin is not favorable under these planctonic growth conditions. Using the same medium to study biofilm production, the transcriptional profile was completely different. Now, the *fyuA* and *iroN* genes were highly upregulated. If this change in iron uptake systems was to satisfy the increased need for iron during biofilm production, you would expect a general upregulation of all iron uptake systems. But the distinct transcriptional profile does not speak in favor of this concept, as *chuA* and *cir* genes were substantially downregulated. The strong expression of FyuA and IroN suggest additional functions of these outer membrane proteins in biofilm, which is apart from just iron acquisition. Transcriptional and translational studies showed a strong expression of IroN and FyuA. In order to investigate the relevance of IroN for biofilm formation, we generated an isogenic *iroN* knockout strain. Growth curves performed in AUM did not reveal limitations of the mutant strain. The loss of IroN resulted in a significant reduction in biofilm production of almost 50% in both UPEC strains NU14 and 536 (data not shown).

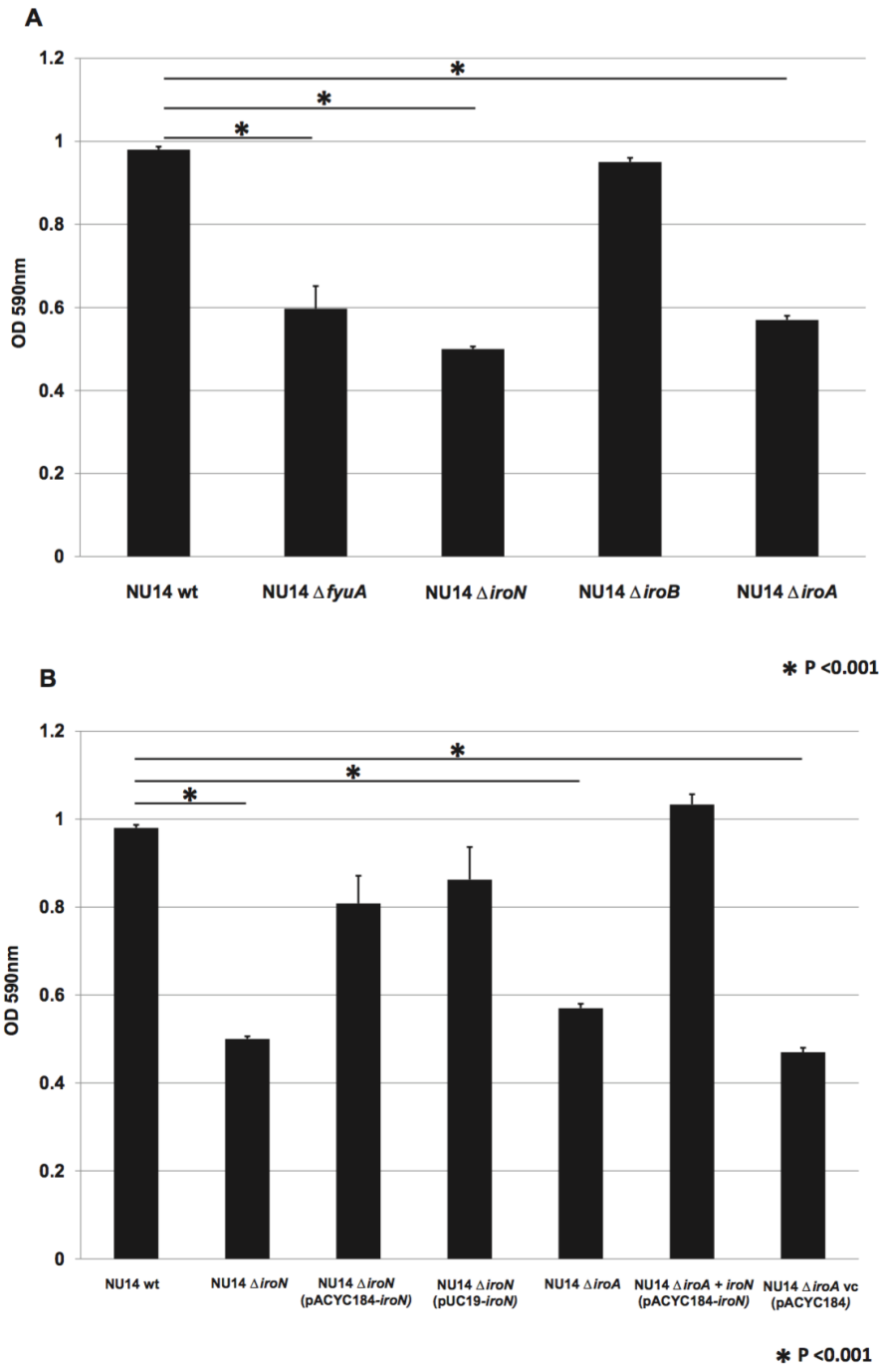

Fig. 3. Loss of FyuA and IroN impairs biofilm formation. (A) Isogenic *fyuA* and *iroN* mutants were tested for biofilm formation. Lane 1: NU14 wild-type, lane 2: NU14 Δ*fyuA*, lane 3: NU14 Δ*iroN*, lane 4: NU14 Δ*iroB*, lane 5: NU14 Δ*iroA*, Both mutants showed a significant reduction in biofilm formation. The isogenic *iroB* mutant displayed no significant change. Deletion of the entire *iroA* locus resulted in a significant decreased biofilm production, indicating that the contribution of the salmochelin system to biofilm formation is mediated by the receptor IroN and not salmochelin synthesis. (B) Impact of IroN expression in salmochelin mutant strains on biofilm formation. Lane 1: NU14 wild-type, lane 2: NU14 Δ*iroN*, lane 3: NU14 Δ*iroN* (pACYC184-*iroN*), lane 4: NU14 Δ*iroN* (pUC19-*iroN*), lane 5: NU14 Δ*iroA*, lane 6: NU14 Δ*iroA* + *iroN* (pACYC184-*iroN*), lane 7: NU14 Δ*iroA* vc (pACYC184, vector control). The *iroN* mutant was complemented with a medium – and high-copy expression plasmid. Biofilm formation was not significantly improved by higher expression of IroN. The complete deletion of the *iroA* locus resulted in a significant reduction in biofilm formation. This deficiency could be restored by complementation with IroN. All experiments were performed in triplicates and repeated at least three times. Error bars represent standard deviations.

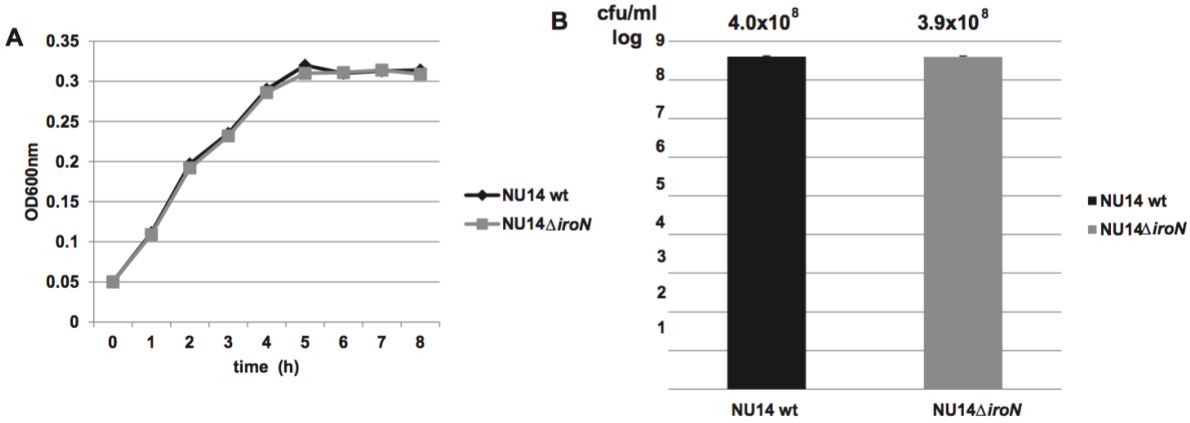

Fig. 4. Growth and final culture density in AUM are not affected in the mutant strain NU14 Δ*iroN*. (A) Growth curves were performed and final culture density (B) was determined in order to evaluate the impact of the deletion of *iroN* on cultivation in AUM. No growth deficiency was observed for the mutant compared to the wildtype strain. Final cell densities of stationary phase cultures (OD_{600nm} = 0.3) were determined. No significant difference between the final cell density (cfu/ml) of NU14 wt and NU14 Δ*iroN* were detected. All experiments were repeated at least three times. Error bars represent standard deviations.

Complementation of the mutant *in trans* restored the capacity to form biofilms. These results provided strong evidence that IroN is required for efficient biofilm formation in UPEC. Of note, the study by Negre et al. (2004) reported previously the contribution of FyuA to biofilm formation, but the microarray analysis performed with an asymptomatic bacteriuria (ABU) strain did not reveal any activation of the salmochelin system at all. Perhaps the *iroA* locus in the tested *E. coli* strain was absent or mutated. Another explanation may be the diversity among UPEC. ABU strains differ considerably in their pathogenicity from virulent UPEC strains and this may also have an influence on the regulation of siderophore systems in biofilms (Klemm et al., 2007). With regard to FyuA our results support the published data (Fig. 3A). In the present study we identified IroN to be a novel determinant of the complex network underlying biofilm formation and we were able to attribute a new function to IroN. It was not clear so far, whether an optimized iron supply is the mechanism or IroN as a single factor, known to have invasive properties, may also display aggregative features. The salmochelin synthesis mutant NU14 Δ*iroB* (Bister et al., 2004; Fischbach et al., 2005; Hantke et al., 2003) showed no significant impairment of biofilm production. A relevant reduction was only observed for the receptor mutant NU14 Δ*iroN*. In biofilms the *iroN* gene is under the control of its own promoter (Hantke et al., 2003; Baumler et al., 1996) and seems to be independently regulated from the *iroBCDE* operon on the polycistronic convergent *iroA* locus. An unknown factor seems to induce the expression of the siderophore receptor IroN and not the production of the siderophore salmochelin. This speaks in favor of a novel function of IroN. With salmochelin being the glycosylated modification of enterobactin (Hantke et al., 2003), IroN might provide stability within biofilms by interacting with glycosylated compounds of the extracellular matrix. The exact mechanism is unknown and warrants further

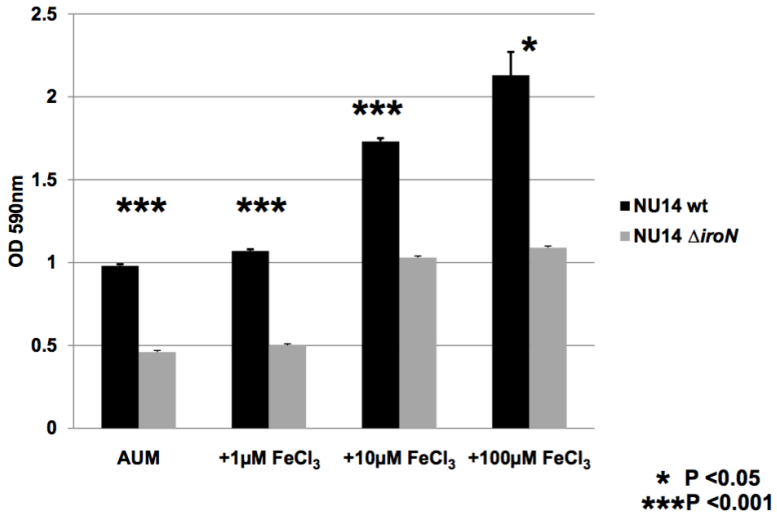

Fig. 5. Influence of iron on biofilm formation. AUM was supplemented with different concentrations of ferric iron (1 μM, 10 μM, 100 μM) and biofilm formation was measured. Rising concentration of additional ferric iron promoted biofilm formation. While 1 μM of $FeCl_3$ had no beneficial effect in NU14 wt and NU14 Δ*iroN*, 10 μM and 100 μM resulted in a 76.5% and 117.3% increase in NU14 wt compared to AUM without iron supplementation, respectively. Also the mutant strain showed complete responsiveness to 10 μM of ferric iron leading to an increase of 123.9%. No further improvement could be achieved by addition of 100 μM ferric iron to AUM in the mutant strain NU14 Δ*iroN*. The significant deficiency in biofilm formation caused by the deletion of *iroN* was stable throughout the experiments.

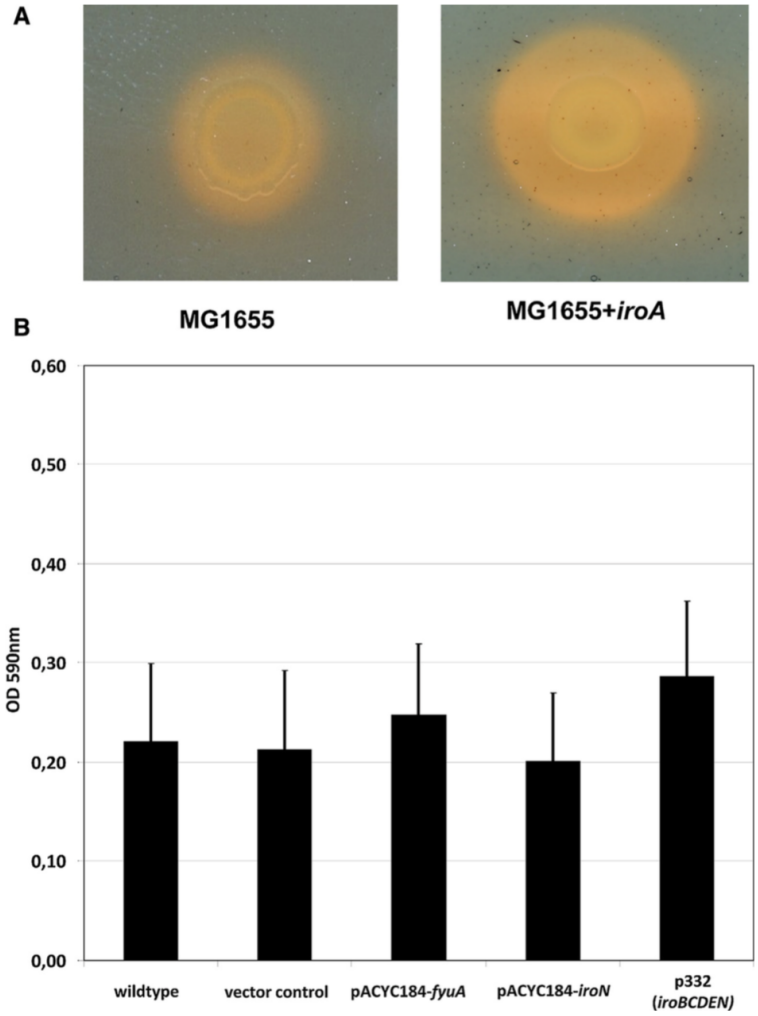

Fig. 6. Neither the additional expression of IroN nor the equipment with a functional salmochelin system improved biofilm formation in *E. coli* K12 strain MG1655. (A) Siderophore production on CAS agar plates. Standardized cultures were spotted on CAS agar plates and the yellow halo around the center indicative for siderophore secretion was determined. The additional production of salmochelin resulted in a larger halo in strain MG1655 + *iroA*. (B). Detection of biofilm production. Lane 1: MG1655 wild-type, lane 2: MG1655 carrying the vector control, lane 3: MG1655 plus pACYC184-*fyuA*, lane 4: MG1655 plus pACYC184-*iroN*, lane 5: MG1655 carrying plasmid p322 (*iroBCDEN*). Neither additional expression of FyuA nor IroN resulted in increased biofilm formation in K12 *E. coli*. The transformation of the functional *iroA* locus did not enhance biofilm formation in AUM. All experiments were performed in triplicates and repeated at least three times. Error bars represent standard deviations.

investigation. The contribution of IroN itself to biofilm formation was supported by our investigation of mutant strain NU14 Δ*iroA*. It lacks the entire salmochelin system. As one might expect, this mutant was also significantly impaired for biofilm production similar to strain NU14 Δ*iroN*. Disruption of the siderophore synthesis in the mutant NU14 Δ*iroB* did not interfere with the ability to form biofilm. The partial complementation of NU14 Δ*iroA* with a plasmid expressing only IroN, restored completely biofilm forming capacity. This outcome corroborates our observation that the outer membrane receptor itself is necessary to promote biofilm formation independently from salmochelin synthesis. We were curious, whether a stronger expression of IroN affects biofilm formation. So we restored *iroN* in the knockout strain with either a medium-copy or a high-copy plasmid. In both cases biofilm production of the mutant equaled wildtype levels. However, we were not able to detect a difference between medium or high expression of IroN. We demonstrated that IroN improves biofilm forming capacity in UPEC, but we wondered whether IroN itself or the entire salmochelin system are able to improve biofilm formation in a poor biofilm producer. We decided to investigate the influence of IroN on biofilm formation in a laboratory *E. coli* K12 strain. This *E. coli* strain MG1655 exhibits a genetic background distinct from UPEC and is known to be a poor biofilm former. Our experiments showed that neither additional expression of IroN nor of FyuA was able to improve biofilm production. Therefore, it appears that the outer membrane proteins alone are not sufficient to promote biofilm formation. Efficient iron uptake by IroN requires the production and secretion of its specific siderophore salmochelin (Hantke et al., 2003). Hence, we introduced the entire *iroBCDEN* locus into *E. coli* K12. The salmochelin system is considered to be an extension to the enterobactin system in *E. coli*, as the chemical modification of enterobactin by glycosylation produces salmochelin (Hantke et al., 2003). Although *E. coli* K12 was equipped with a functional salmochelin system, we did not detect any improvement in biofilm formation. At least, the idea of salmochelin as "biofilm-specific" siderophore to meet the increased needs for iron in biofilms can

be excluded. The contribution of the salmochelin receptor IroN to biofilm formation cannot be explained by a mechanism involving the uptake of ferric iron *via* salmochelin for the following reasons. First of all, deletion of *iroN* hardly impairs the synthesis and secretion of the catecholate siderophore salmochelin. It has even been demonstrated that the lack of *iroN* results in increased production of linear salmochelin (S2) (Caza et al., 2008). Uptake experiments with radiolabeled ^{55}Fe-salmochelins proved that alternative catecholate receptors like the enterobactin receptor FepA and the outer membrane receptor Cir are able to take up salmochelins (Hantke et al., 2003). This is relevant for the interpretation of our presented results. FepA and Cir are present in the mutant strain NU14 Δ*iroN* and are also expressed by laboratory *E. coli* K12 strain MG1655. This implies that these compounds still can be utilized by NU14 Δ*iroN*. Thus, salmochelin-mediated iron uptake is still possible. Second, excessive expression of IroN in the mutant strain NU14 Δ*iroN* is supposed to optimize salmochelin-mediated iron uptake. Assuming a salmochelin-dependent mechanism for biofilm formation, this should increase biofilm production. But this was not the case. As mentioned above, salmochelins can be taken up by FepA and Cir. Both receptors are expressed by laboratory *E. coli* K12 strain MG1655. Complemented with the entire functional *iroA* locus, additional iron uptake is achieved. However, enhanced iron uptake did not lead to increased biofilm production in *E. coli* K12. Furthermore, investigation of the influence of different concentration of ferric iron on biofilm production revealed complete responsiveness to iron supplementation in the mutant strain NU14 Δ*iroN*. The deficiency regarding biofilm formation compared to the wild-type strain was stable throughout the experiments. Our findings are in line with the growing body of evidence on the role of IroN for virulence. Production and secretion of salmochelins are hardly affected in a Δ*iroN* mutant strain, but IroN itself is required for virulence (Negre et al., 2004; Russo et al., 2002; Caza et al., 2008). The exact mechanism still remains elusive so far. In conclusion, here we identified IroN as a new factor relevant for efficient biofilm formation in UPEC. This effect is independent from salmochelin-mediated iron supply. It seems that a certain genetic background found in UPEC and not in *E. coli* K12 is required for this phenotype. This concept is supported by our results. Promotion of biofilm formation is achieved through the expression of IroN in the UPEC strain NU14 Δ*iroA*, lacking the entire salmochelin system, but not in *E. coli* K12. Stronger expression of IroN does not enhance the biofilm forming capacity of UPEC in general. Further experiments are necessary to elucidate the exact molecular mechanism of IroN in biofilms. It may interact with components of the extracellular matrix in biofilms or other components of the outer membrane. This work highlights the multi-functional role of IroN and supports the concept of a complex but orchestrated network of virulence and fitness factors contributing to the pathogenicity of ExPEC.

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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 interchangeability 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 phosphopantetheinyl-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.

Citation: Martin P, Marcq I, Magistro G, Penary M, Garcie C, et al. (2013) Interplay between Siderophores and Colibactin Genotoxin Biosynthetic Pathways in *Escherichia coli*. PLoS Pathog 9(7): e1003437. doi:10.1371/journal.ppat.1003437

Editor: Xavier Nassif, Faculté de Médecine Paris Descartes, site Necker, France

Received February 19, 2013; **Accepted** May 2, 2013; **Published** July 11, 2013

Funding: This research was supported by grants from FEDER (MYCA), from the Centre de Physiopathologie de Toulouse Purpan (CPTP, Sepsis: the critical role of iron) and from the French National Research Agency (ANR). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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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 salmochelins production is located either on a chromosomal pathogenicity island or on a transmissible plasmid [16]. Contrary to enterobactin, salmochelins are able to evade the

Author Summary

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 interchangeability 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 (siderocalin) 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 phylogenetic 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 was 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

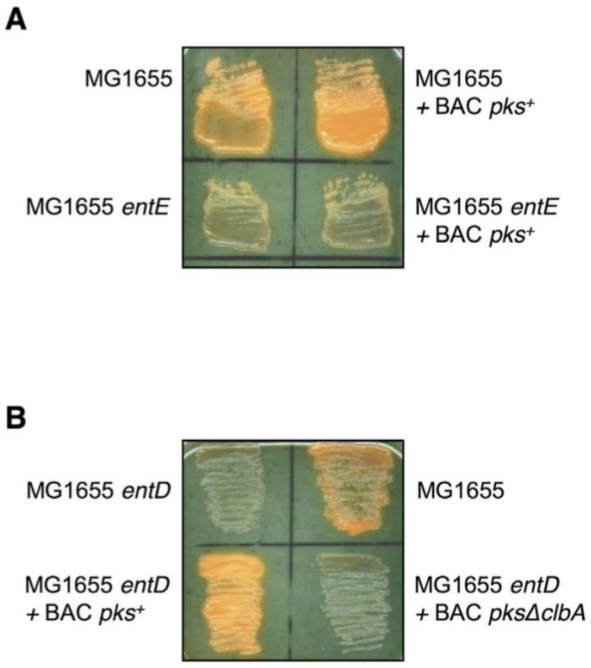

Figure 1. Siderophore production by *Escherichia coli* strain MG1655 and derivatives. Chrome azurol S (CAS) plates upon which the *E. coli* strain MG1655 and derivatives have been grown overnight. **A.** Wild type and *entE* derivatives of strain MG1655. **B.** Wild type and *entD* derivatives of strain 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.
doi:10.1371/journal.ppat.1003437.g001

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 pigmentation 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 *entD*+BAC *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 ClbA 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 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 resulted in the abrogation of the production of siderophores in strain SE15 (Fig. 2A and 2B). As expected, complementation with *entD* 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 *ybtD* gene that encodes the YbtD 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 *ybtD*, *pptT* and *sfp* genes.

These data evidenced that ClbA can be xeno-complemented for the colibactin synthesis.

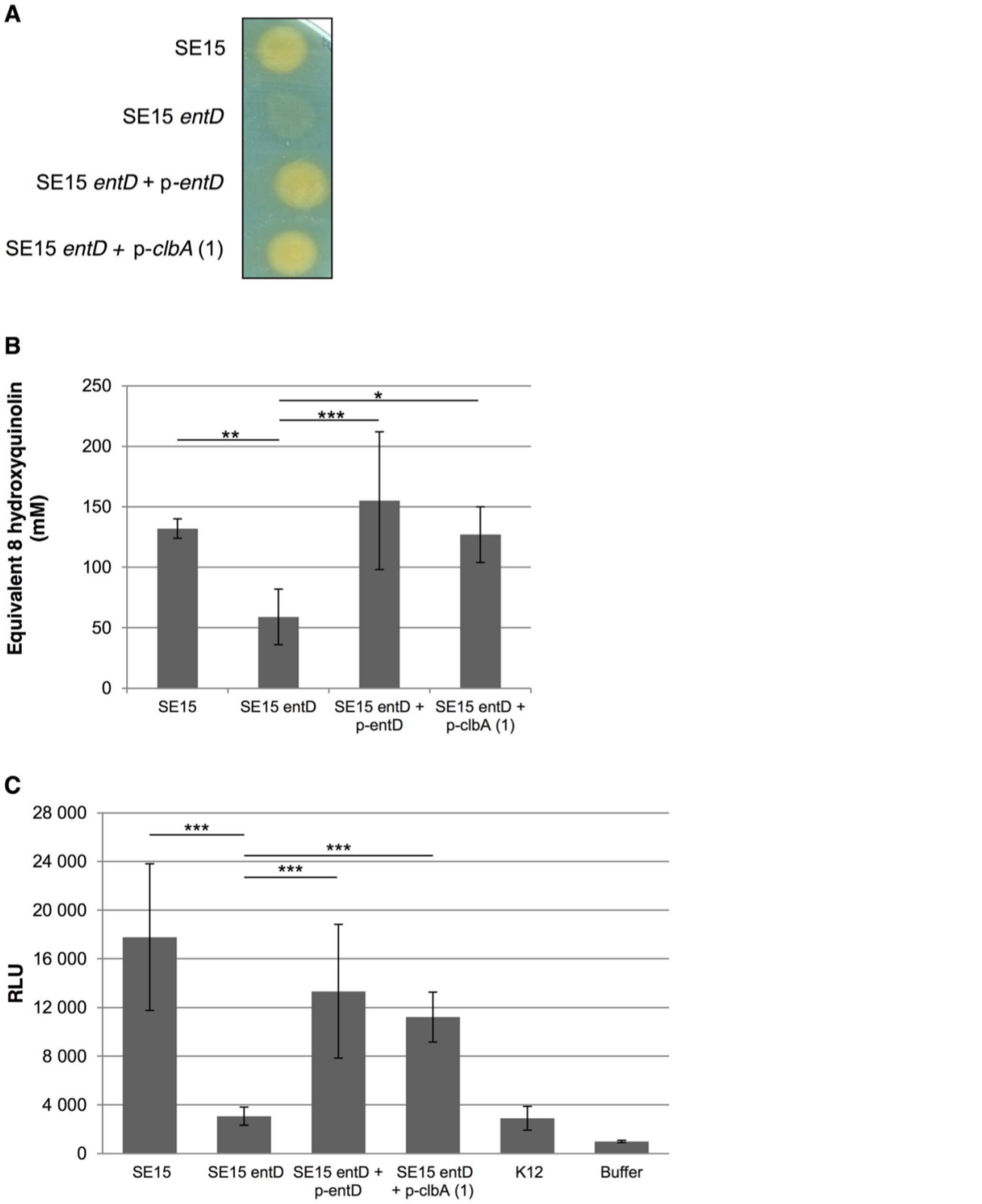

Figure 2. Both EntD and ClbA can support the yersiniabactin siderophore synthesis *in vitro*. Siderophore production by the enterobactin and yersiniabactin siderophores producer *Escherichia coli* strain SE15 and derivatives. **A.** Chrome azurol S (CAS) plate upon which *E. coli* strain SE15 and derivatives 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. ***: $P<0.001$, **: $P<0.01$, *: $P<0.05$.
doi:10.1371/journal.ppat.1003437.g002

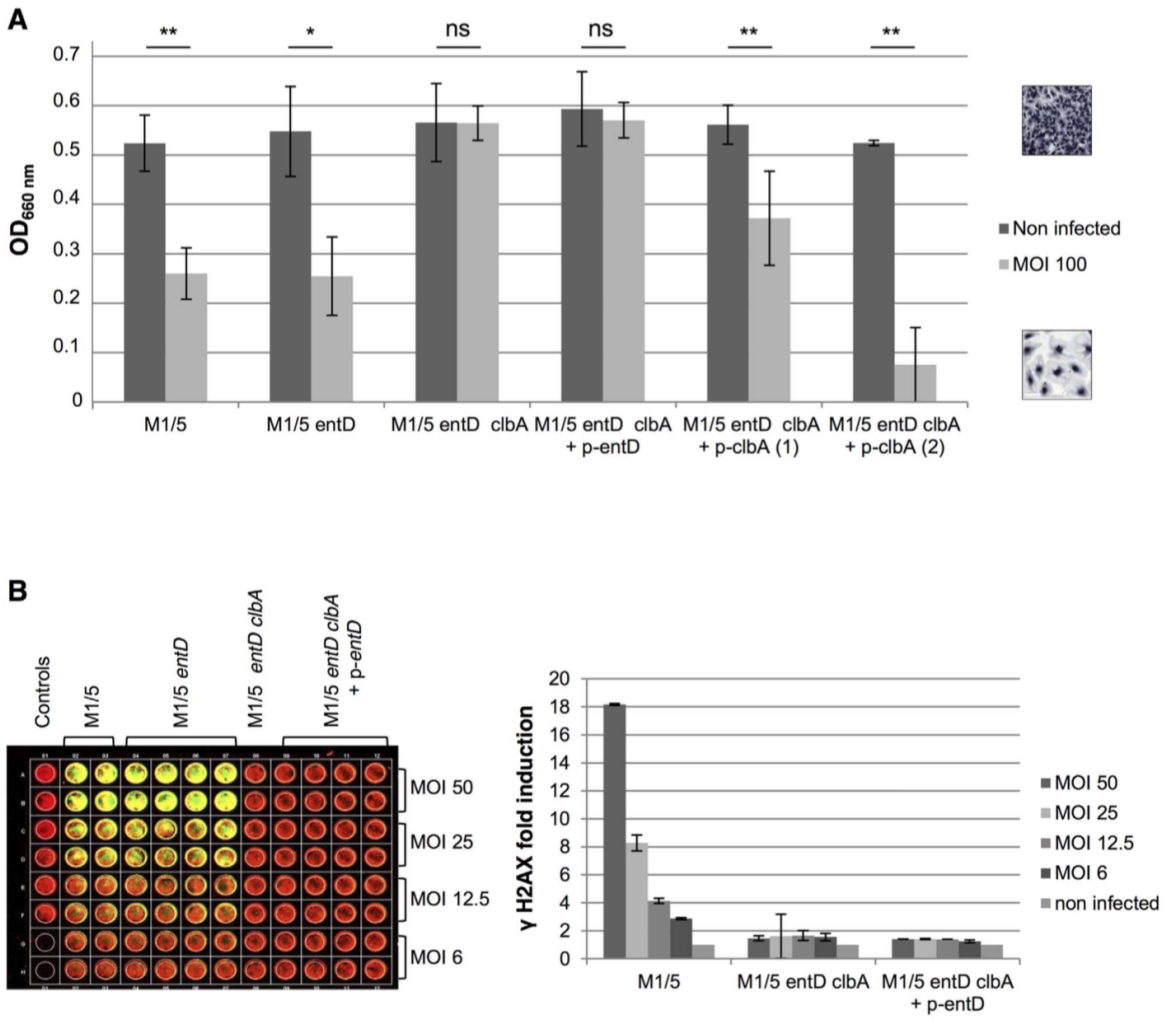

Figure 3. Colibactin synthesis cannot be sustained by EntD *in vitro*. Colibactin production by *Escherichia coli* strain M1/5 and derivatives determined by megalocytosis (**A**) and by quantification of DNA double strand breaks (**B**) in infected HeLa cells. **A.** Live *E. coli* wild type strain M1/5, mutants and complemented derivatives were added directly onto HeLa cells [multiplicity of infection (MOI) = 100], cocultivated for 4 h, then washed as described in Nougayrède et al. [21]. The cells were incubated for 72 h with gentamicin before protein staining with methylene blue. The quantification of staining was measured at OD 660 nm. **: $P<0.01$, *: $P<0.05$, ns: not significant. **B.** Quantification of DNA double strand breaks through the quantification of phosphorylated H2AX (γ-H2AX) using In Cell Western method [27]. HeLa cells were infected 4 h with strain M1/5 and derivatives [MOI = 50 to 6] fixed, and examined 8 h post infection for quantification of γ-H2AX.
doi:10.1371/journal.ppat.1003437.g003

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 (indigoidine), from a single substrate (L-glutamine) [31]. Plasmid p-*BpsA* that encodes BspA was transformed into strain MG1655 *entD*. The resulting MG1655 Δ*entD*+p-*BspA* strain was subsequently transformed with plasmids carrying *ybtD*, *pptT*, *sfp*, *clbA*, or *entD* genes. In addition, *E. coli* strain MG1655 BAC *pks*$^{+}$ and MG1655 BAC *pksΔclbA* 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 Δ*entD*+p-*BspA*+p-*entD* (Fig. 5A). A quantification of the indigoidine 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

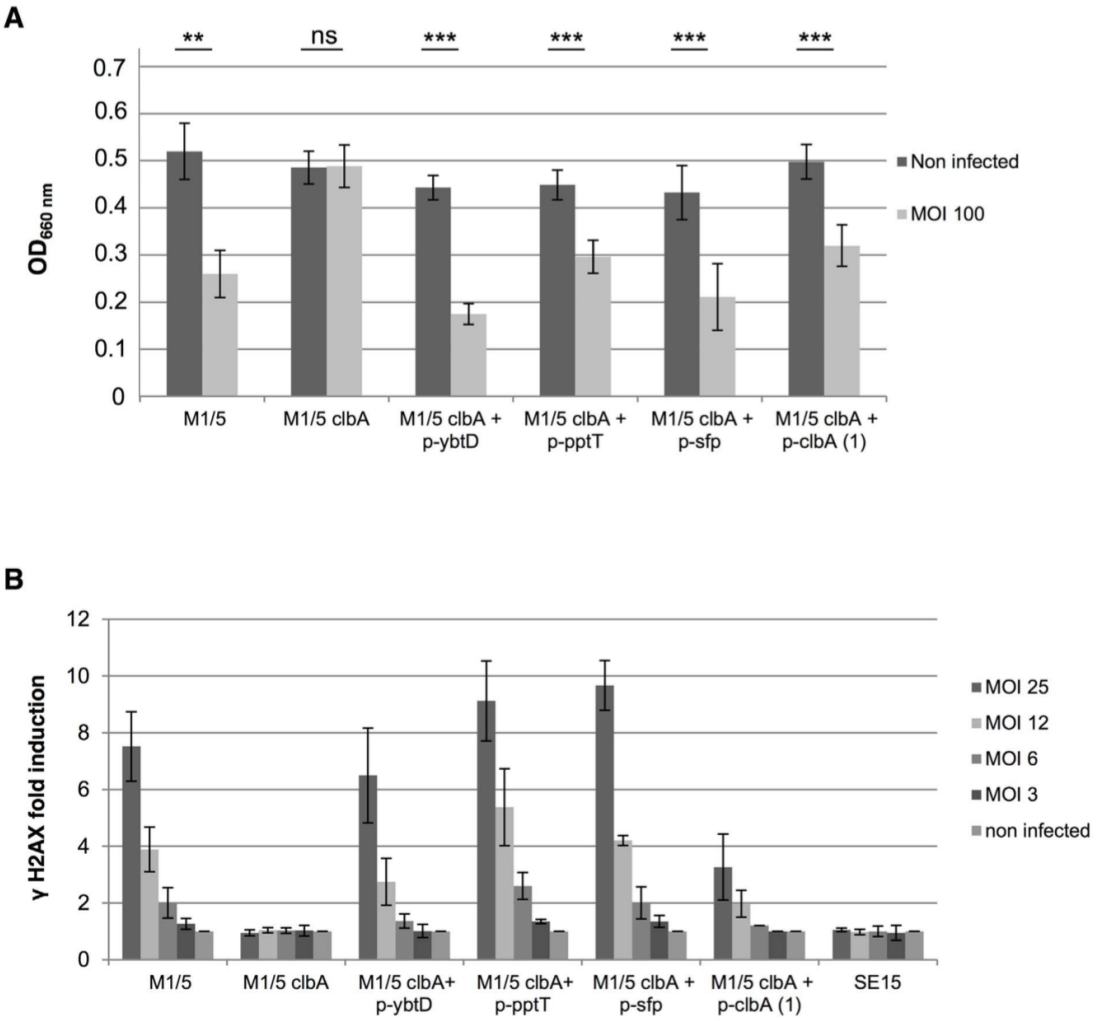

Figure 4. Colibactin synthesis can be sustained by exogenous PPTases *in vitro*. Colibactin production by *Escherichia coli* strain M1/5 and derivatives determined by megalocytosis (**A**) and by quantification of DNA double strand breaks (**B**), as in Fig. 3. ***: $P<0.001$, **: $P<0.01$, ns: not significant. *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*.
doi:10.1371/journal.ppat.1003437.g004

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*. No bacteria were recovered from spleen or blood of mice injected with the double mutant SP15 *entD clbA* (Fig. 6B).

This demonstrated that both EntD and ClbA must be inactivated to abolish virulence of ExPEC in a mouse model of sepsis.

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

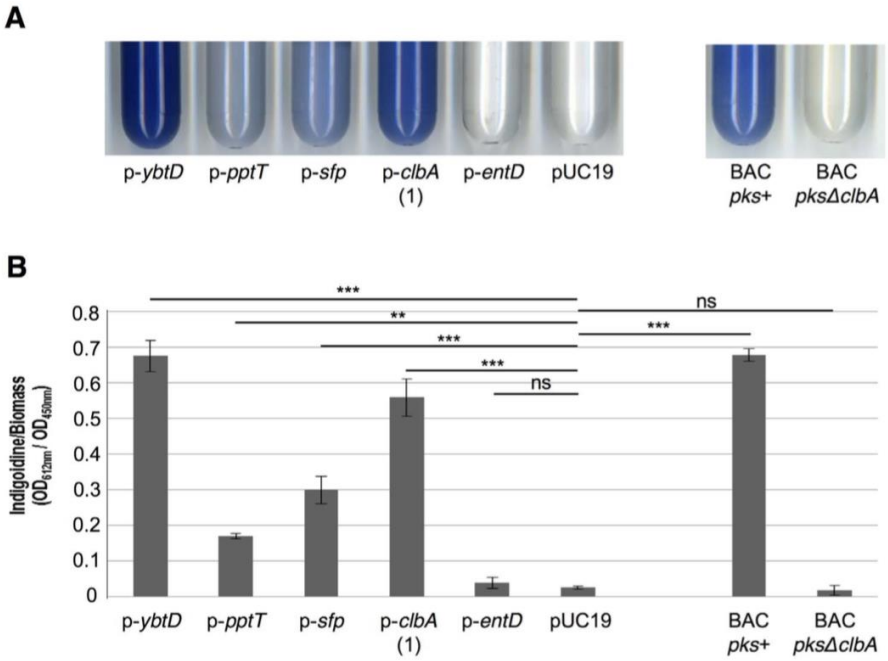

Figure 5. ClbA is more promiscuous in its substrate specificity than EntD. The synthesis of the single-module non-ribosomal peptide synthetase BpsA from *Streptomyces lavendulae* resulting in the production of indigoidine was qualitatively (**A**) and quantitatively (**B**) assessed as previously described [31,51]. *E. coli* strain MG1655 Δ*entD*+p-*BpsA* was transformed with the plasmids p-*ybtD*, p-*pptT*, p-*sfp*, p-*clbA* (1), p-*entD* and pUC19 (left). *E. coli* strain MG1655 BAC *pks*$^+$ and MG1655 BAC *pksΔclbA* were transformed with p-*BpsA* (right). **A.** All the strains were grown overnight at 18°C in an auto-inducer medium. The cultures were left at room temperature so that the blue pigmentation (indigoidine) is produced. **B.** Quantification of indigoidine in the different strains. The data are the means and standard deviations of 3 independent experiments. ***: $P<0.001$, **: $P<0.01$, ns: not significant.
doi:10.1371/journal.ppat.1003437.g005

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

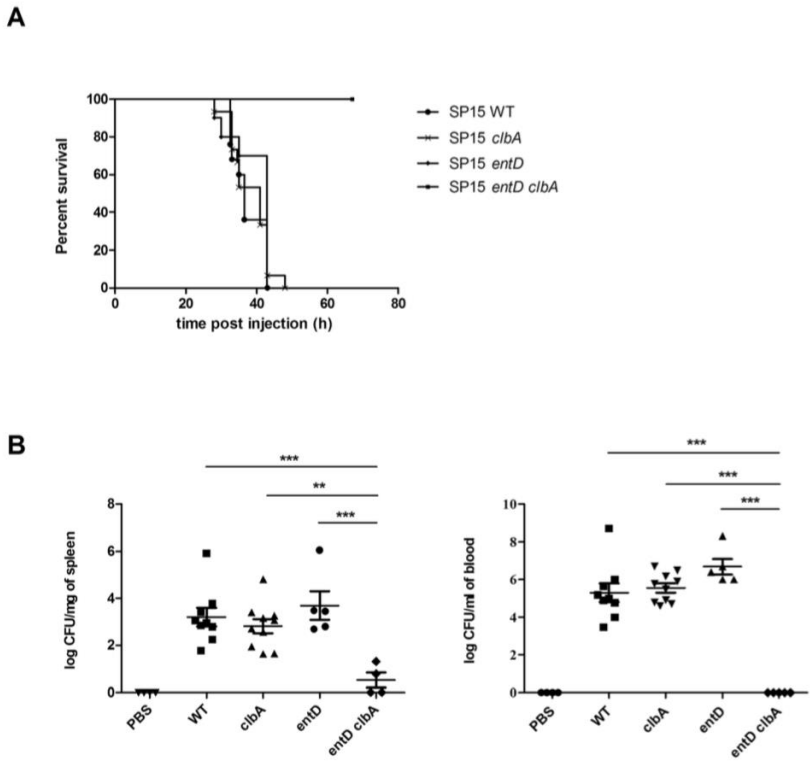

Figure 6. Both EntD and ClbA must be inactivated to abolish virulence of ExPEC. Mice underwent footpad injection with 10^8 CFU of *E. coli* SP15 wild type strain or derivatives. **A.** The percentage of mice survival was monitored. 10 to 25 mice were used per group. **B.** 18 h post infection 4 to 10 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. ***: $P<0.001$, **: $P<0.01$.
doi:10.1371/journal.ppat.1003437.g006

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

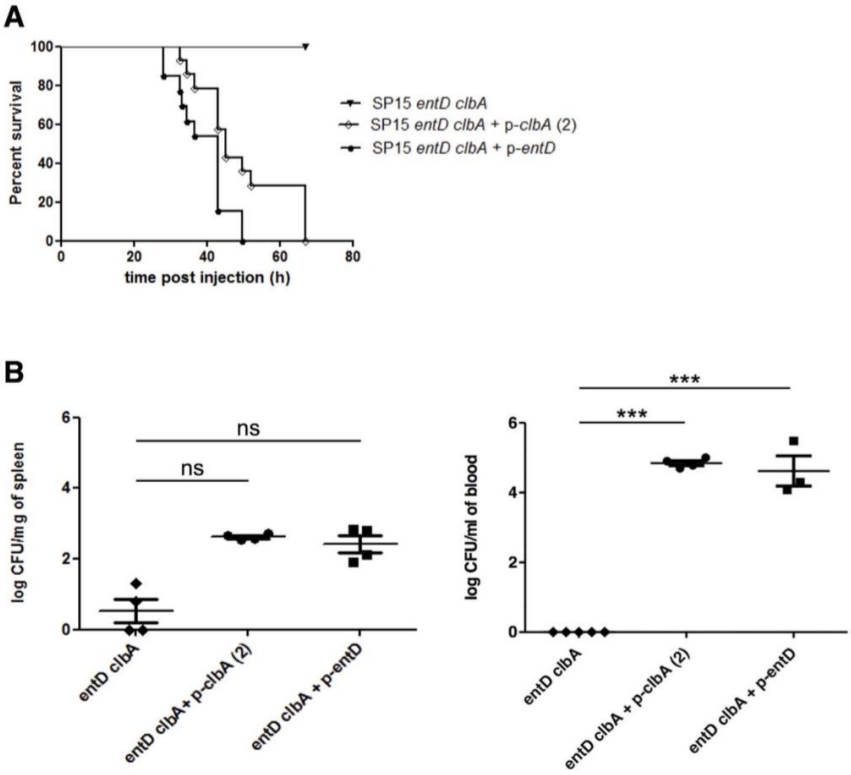

Figure 7. The presence of either EntD or ClbA is required to maintain full virulence of ExPEC. Mice underwent footpad injection with 10^8 CFU of *E. coli* SP15 *entD clbA* strain and complemented derivatives. **A.** The percentage of mice survival was monitored. 10 to 25 mice were used per group. **B.** 18 h post infection 3 to 5 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. ***: $P<0.001$, ns: not significant.
doi:10.1371/journal.ppat.1003437.g007

enterobactin and consequently salmochelins). This deadly association is not confined in *E. coli*. Similar events also occurred in other pathogenic *Enterobacteriacae* 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 pBR322 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 $OD_{630\ nm}$. For quantification, the iron chelating agent 8 hydroxyquinoline (8HQ, sigma-aldrich) was used as the standard.

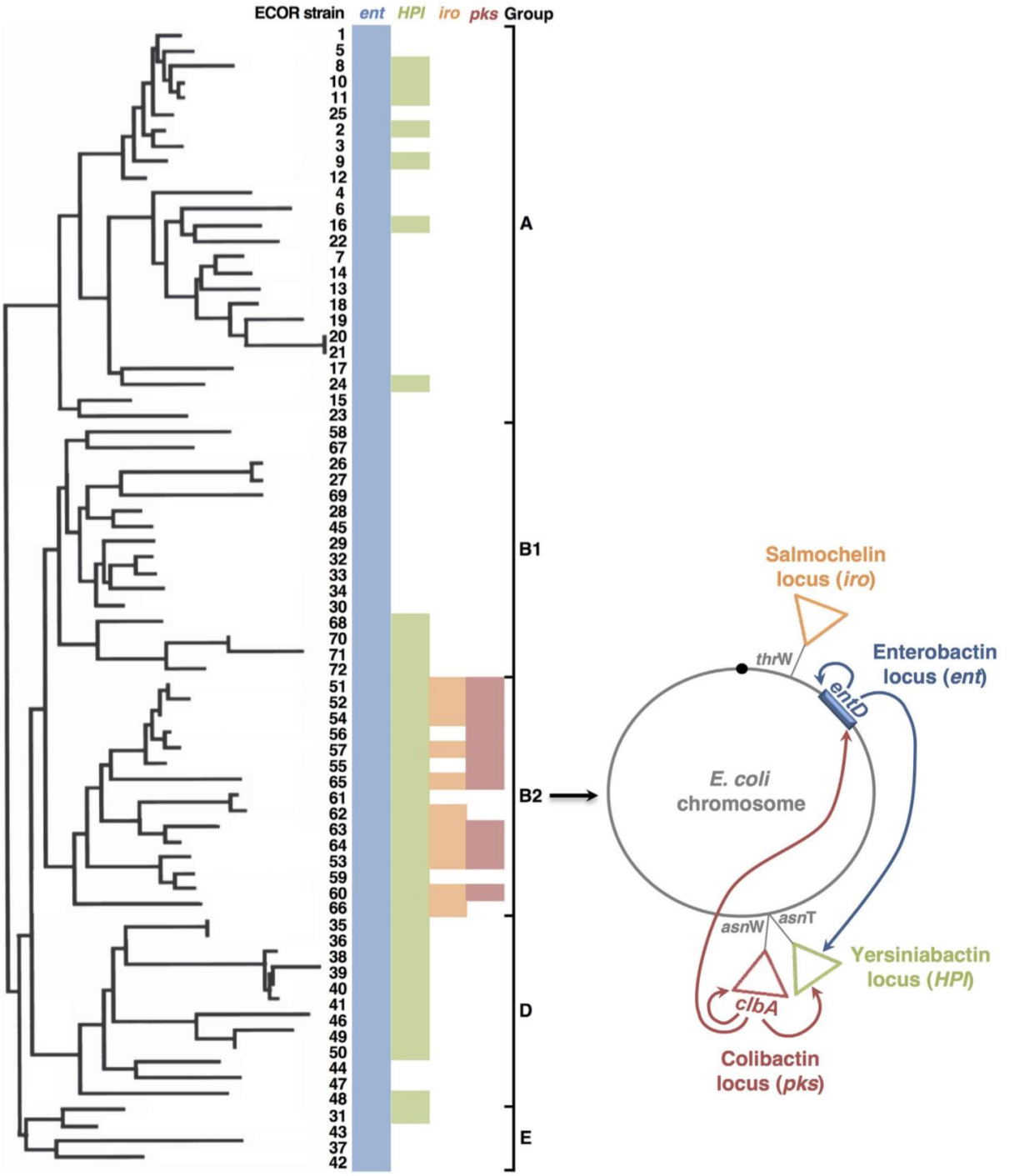

Figure 8. Model for the co-evolution of the *pks* and high pathogenicity islands in *E. coli*. Left. Phylogenetic relationships amongst the *E. coli* reference strains (ECOR, [3,52]). The phylogeny was based on MLST of back-bone genes [3,43]. Ent locus positive strains are indicated in blue, HPI island positive strains are indicated in green [43], iro locus positive strains are indicated in orange and *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 *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.
doi:10.1371/journal.ppat.1003437.g008

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^r, Ap^r, Kan^r, Cm^r; *fepA*::Tn*10d*Tc, *iroN*::pGP704, *cir*::MudJ carrying pACYC5.3L plasmid	Gift from W. Rabsch
MG1655 *entE*	*entE* mutant of strain MG1655; Kan^r	This study
MG1655 *entE*+BAC *pks+*	*entE* mutant of strain MG1655 carrying BAC *pks+*; Kan^r, Cm^r	This study
MG1655 *entD*	*entD* mutant of strain MG1655; Kan^r	This study
MG1655 *entD*+BAC *pks+*	*entD* mutant of strain MG1655 carrying BAC *pks+*; Kan^r, Cm^r	This study
MG1655 *entD*+BAC *pksΔclbA*	*entD* mutant of strain MG1655 carrying BAC *pksΔclbA*; Kan^r, Cm^r	This study
MG1655 *entD*+p-*clbA* (1)	*entD* mutant of strain MG1655 carrying p-*clbA* (1); Kan^r Amp^r	This study
MG1655 Δ*entD*	*entD* mutant of strain MG1655	This study
MG1655 Δ*entD*+p-*bpsA*	*entD* mutant of strain MG1655 carrying p-*bpsA*; Kan^r	This study
MG1655 Δ*entD*+p-bpsA+p-*entD*	*entD* mutant of strain MG1655 carrying p-*bpsA* and p-*entD*; Kan^r Amp^r	This study
MG1655 Δ*entD*+p-*bpsA*−+p-*clbA* (1)	*entD* mutant of strain MG1655 carrying p-*bpsA* and p-*clbA* (1); Kan^r Amp^r	This study
MG1655 Δ*entD*+p-*bpsA*+p-*sfp*	*entD* mutant of strain MG1655 carrying p-*bpsA* and p-*sfp*; Kan^r Amp^r	This study
MG1655 Δ*entD*+p-*bpsA*+p-*pptT*	*entD* mutant of strain MG1655 carrying p-*bpsA* and p-*pptT*; Kan^r Amp^r	This study
MG1655+BAC *pks+*	strain MG1655 carrying BAC *pks+*; Cm^r	This study
MG1655+BAC *pksΔclbA*	strain MG1655 carrying BAC *pksΔclbA*; Cm^r	This study
MG1655+BAC *pks+*+p-*bpsA*	strain MG1655 carrying BAC *pks+* and p-*bpsA*; Kan^r Cm^r	This study
MG1655+BAC *pksΔclbA*+p-*bpsA*	strain MG1655 carrying BAC *pksΔclbA* and p-*bpsA*; Kan^r Cm^r	This study
SE15	Enterobactin and yersiniabactin siderophores producer	[46]
SE15 *entD*	*entD* mutant of strain SE15; Kan^r	This study
SE15 *entD*+p-*entD*	*entD* mutant of strain SE15 carrying p-*entD* plasmid; Kan^r Amp^r	This study
SE15 *entD*+p-*clbA* (1)	*entD* mutant of strain SE15 carrying p-*clbA* (1) plasmid; Kan^r Amp^r	This study
M1/5	Enterobactin, aerobactin and yersiniabactin siderophores producer	Gift from U. Dobrindt
M1/5 *entD*	*entD* mutant of strain M1/5; Kan^r	This study
M1/5 *clbA*	*clbA* mutant of strain M1/5; Kan^r	This study
M1/5 *entD clbA*	*entD clbA* mutant of strain M1/5; Kan^r	This study
M1/5 *entD clbA*+p-*entD*	*entD clbA* mutant of strain M1/5 carrying p-*entD*; Kan^r Amp^r	This study
M1/5 *entD clbA*+p-*clbA* (1)	*entD clbA* mutant of strain M1/5 carrying p-*clbA* (1); Kan^r Amp^r	This study
M1/5 *entD clbA*+p-*clbA* (2)	*entD clbA* mutant of strain M1/5 carrying p-*clbA* (2); Kan^r Amp^r	This study
M1/5 *clbA*+p-*clbA* (1)	*clbA* mutant of strain M1/5 carrying p-*clbA* (1); Kan^r Amp^r	This study
M1/5 *clbA*+p-*sfp*	*clbA* mutant of strain M1/5 carrying p-*sfp* plasmid; Kan^r Amp^r	This study
M1/5 *clbA*+p-*pptT*	*clbA* mutant of strain M1/5 carrying p-*pptT* plasmid; Kan^r Amp^r	This study
SP15	Enterobactin, salmochelin, aerobactin and yersiniabactin siderophores producer	[53]
SP15 *clbA*	*clbA* mutant of strain SP15; Kan^r	This study
SP15 *entD*	*entD* mutant of strain SP15; Kan^r	This study
SP15 *entD clbA*	*entD clbA* mutant of strain SP15; Kan^r	This study
SP15 *entD clbA*+p-*clbA* (2)	*clbA* mutant of strain SP15 carrying p-*clbA* (2); Kan^r Amp^r	This study
SP15 *entD clbA*+p-*entD*	*entD clbA* mutant of strain SP15 carrying p-*entD*; Kan^r Amp^r	This study
Plasmids		
pACYC5.3L	*fyuA-*, *ybtA-*, *fyuA-luc-*, *irp6-8*, Cm^r	Gift from W. Rabsch
p-*entD*	High copy number PCR-Script plasmid carrying *entD* gene; Amp^r	This study
p-*clbA* (1)	Low copy number pASK75 plasmid carrying *clbA* gene; Amp^r	Gift from U. Dobrindt
p-*clbA* (2)	pMB808, high copy number PCR-Script plasmid carrying *clbA* gene; Amp^r	[22]
BAC *pks+*	Bacterial artificial chromosome carrying the entire *pks* island; Cm^r	[21]
BAC *pksΔclbA*	Bacterial artificial chromosome carrying the entire *pks* island with deleted *clbA* gene; Cm^r	[22]

Table 1. Cont.

Strain or plasmid	Genotype or phenotype	Source or reference
p-*sfp*	Low copy number pET26b plasmid carrying *sfp* gene from *Bacillus subtilis*; Amp^r	Gift from C. Chalut
p-*pptT*	Low copy number pET28a plasmid carrying *pptT* gene from *Mycobacterium tuberculosis*; Amp^r	Gift from C. Chalut
p-*bpsA*	Low copy number pET26b plasmid carrying gene *bpsA* from *Streptomyces lavendulae*, Kan^r	Gift from C. Chalut

doi:10.1371/journal.ppat.1003437.t001

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 α,α'-dipyridyl (Sigma), for 24 h at 37°C. Bacteria were pelleted by centrifugation and the supernatant was added to the indicator strain WR1542 carrying plasmid pACYC5.3L (kind gift of W. Rabsch, Wernigerode). The plasmid encodes all genes necessary for yersiniabactin uptake; *i.e. irp6*, *irp7*, *irp8*, *fyuA* and *ybtA*. Additionally, the *fyuA* promoter region fused to the luciferase reporter gene is included on pACYC5.3L. After further 24 h of incubation at 37°C the indicator strain was pelleted and resuspended in bacterial lysis buffer (100 mM potassium phosphate buffer [pH 7.8], 2 mM EDTA, 1% [wt/vol] Triton X-100, 5 mg/ml bovine serum albumin, 1 mM dithiothreitol, 5 mg/ml lysozyme). Complete lysis was performed by incubation at room temperature for 20 min and repeated mixing. The samples were centrifuged and supernatants of lysates were analyzed by addition of luciferase reagent (20 mM Tricine-HCl (pH 7.8), 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2$, 100 µM EDTA, 470 µM D(-) luciferin, 33.3 mM dithiothreitol, 270 µM Li_3 coenzyme A, 530 µM Mg-ATP). Luciferase activities were determined in triplicates using the multimode reader Berthold Tristar LB 941. Values were corrected by relating luciferase activity to the OD_{600} of bacterial cultures grown 24 h in NBD medium. K12 *E. coli* strain DH5α served as negative control. The experiments were repeated at least three times.

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 [51]. 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 ($OD_{450\ nm}$). Finally, the indigoidine production was normalized with the ratio Indigoidine/Biomass (*e.g.* ratio $OD_{612\ nm}/OD_{450\ nm}$).

Determination of the megalocytosis induced by colibactin

HeLa cells were maintained by serial passage in DMEM supplemented with 10% FCS, non-essential amino acids and 50 µg/mL gentamicin. HeLa cells were dispensed in 96-well cell culture plate (5×10^3 cells/well). For bacterial infections, overnight LB broth cultures of *E. coli* were diluted in interaction medium (DMEM, 5% FCS, 25 mM HEPES) and cell cultures (~70% confluent) were infected with a multiplicity of infection (number of bacteria per HeLa cell at the onset of infection) of 3 to 400. Four hours post-inoculation, cells were washed 3 times with HBSS and incubated in cell culture medium 72 h with 200 µg/mL gentamicin before protein staining with methylene blue (1% w/v in Tris-HCl 0.01M). The methylene blue was extracted with HCl 0.1N. The quantification of staining was measured at $OD_{660\ nm}$.

Determination of the genotoxic effect induced by colibactin

The In Cell Western procedure was performed as described previously [27]. Briefly, HeLa cells were dispensed in 96-well cell culture plate (1.5×10^5 cells/200 µL/wells). Twenty four hours later, cells were infected with *E. coli* strains for 4 h. Eight hours post-infection the cells were directly fixed in the plate with 4% paraformaldehyde. Paraformaldehyde was neutralized, and cells were permeabilized as previously described [27]. Cells were blocked with MAXblock Blocking medium (Active Motif,

Table 2. Oligonucleotides used in the study.

Primers	Sequences
entD-P1	GGGCGGATCGCTGCAATTTATGCGCTGCGGGAATATGGCTATAAATGTGTGCGTGTAGGCTGGAGCTGCTTC
entD-P2	TCACTTGCCTTAAATGCGCTCTCTTTGGCGGAAAATGCCAGTGTCAGCGCCATATGAATATCCTCCTTAG
entD-Up	CCCCCGGGGGGGACGTACGTGGTATATGAGC
entD-Down	AACTGCAGAAGCACCTGCTTTACACTTTCG
entE-P1	TATCGACGGCGAGCGACAGTTGAGTTATCGGGAGCTGAATCAGGCGGCGTGTAGGCTGGAGCTGCTTC
entE-P2	AAGCGCAGCTTTTTTCGCCCATCAGCTCATCTTCCATGCTCACCAGTGCCATATGAATATCCTCCTTAG
JPN42	CAG ATA CAC AGA TAC CAT TCA
JPN46	CTA GAT TAT CCG TGG CGA TTC

doi:10.1371/journal.ppat.1003437.t002

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 (IRDyeTM 800CW, 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 experimentations 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 Ethique 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/6J mice (JANVIER) were injected into the footpad with 10^8 ExPEC WT, *clbA* mutants, *entD* mutant, *entD clbA* mutant and *entD clbA* mutant complemented with *clbA* (*entD clbA*+p-*clbA*(2)) and *entD* (*entD clbA*+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 0.05 was considered statistically significant and is denoted by *. $P<0.01$ is denoted by ** and $P<0.001$ by ***.

Acknowledgments

We thank Dr. Damien Dubois for the *in silico* analysis of conserved domains of PPTases and for critical reading of the manuscript. We thank Claude Watrin for excellent technical help.

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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7. Danksagung

Für die Möglichkeit am Max von Pettenkofer-Institut promovieren zu dürfen bedanke ich mich bei Herrn Prof. Dr. med. Dr. rer. nat. Jürgen Heesemann und seinem Nachfolger Prof. Dr. med. Sebastian Suerbaum.

Für die Überlassung des Themas, die Freiheit selbständig forschen zu dürfen, die unzähligen Gelegenheiten an nationalen und internationalen Kooperationen mitwirken zu dürfen und die außerordentliche Betreuung und Unterstützung gilt mein aufrichtiger Dank Herrn Prof. Dr. med. Sören Schubert.

Schließlich möchte ich mich bei allen Doktoranden, MTAs, Postdocs, und Arbeitsgruppenleitern am Max von Pettenkofer-Institut und Kooperationsinstituten für die Unterstützung im Rahmen gemeinsamer Projekte bedanken.