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Abstract

The genotoxin colibactin is a secondary metabolite produced by a variety of pathogenic Enterobacteria, and is associated with colon cancer development and acute systemic infections. The colibactin biosynthesis requires the enzymatic activity of the phosphopantetheinyl transferase ClbA. We recently evidenced that two master regulators of bacterial iron homeostasis, *i.e.* the ferric uptake regulator (Fur) and the small regulatory non-coding RNA RyhB, were involved in the regulation of the *clbA* transcription and of the colibactin production. In this study we investigated the impact of high iron supply on *clbA* transcription and colibactin production in wild type, Δ*ryhB*, Δ*fur* and Δ*ryhB* Δ*fur* strains. This revealed that high iron resulted in decreased synthesis of the genotoxin colibactin through both pathways dependent and independent of Fur/RyhB. This work highlights the complex regulatory mechanism that controls an important bacterial virulence and carcinogenesis factor by regulators of bacterial iron homeostasis.

Introduction

The regulation of iron homeostasis in bacteria mainly relies on ferric uptake regulator (Fur) protein and the small regulatory non-coding RNA (sRNA) RyhB (Massé *et al*. 2007; Troxell and Hassan 2013; Porcheron and Dozois 2015). Fur is a global transcriptional regulator that controls the transcription of over 90 genes involved in iron uptake, storage and metabolism. Fur is a well-known repressor. However, a growing literature reports Fur as an activator (Yu and Genco 2012; Troxell and Hassan 2013). Fur has been shown to play an important role in the modulation of bacterial virulence (Gancz *et al*. 2006; Payne *et al*. 2006; Ledala *et al*. 2010; Torres *et al*. 2010; Tobe *et al*. 2014). Bacterial small regulatory RNA (sRNAs) are commonly known to repress gene expression by base pairing to target mRNAs. In contrast, a growing number of examples of translational activation and mRNA stabilization by sRNAs have now

been documented (Papenfort and Vanderpool 2015). Although Fur and iron directly regulate sRNA RyhB (Massé *et al*. 2005; Sobrero and Valverde 2012), strong evidence suggests that RyhB itself is sufficient to influence the level of free intracellular iron (Massé *et al*. 2005). RyhB was shown to be implicated in virulence-associated processes in pathogenic bacteria such as *Shigella flexneri* (Oglesby *et al*. 2005), *S. dysenteriae* (Murphy and Payne 2007), *Listeria monocytogenes* (Sesto *et al*. 2014) or *Escherichia coli* (Porcheron *et al*. 2014).

It is observed over the last 30 years a change in the composition of commensal *E. coli* population in industrialized countries, with an increase in the phylogenetic group B2 (Tenaillon *et al*. 2010; Massot *et al*. 2016). Genetic analysis of different *E. coli* strains showed that those of the phylogenetic group B2 have developed a greater ability to acquire iron (Schubert *et al*. 2009). Colibactin is a toxin synthesized by a diversity of pathogenic Enterobacteria, including approximately 50% of the *E. coli* stains that belong to the phylogenetic group B2 (Nougayrède *et al*. 2006). This genotoxin induces DNA double strand breaks, senescence, chromosomal abnormalities in enterocytes both *in vitro* and *in vivo* (Cuevas-Ramos *et al*. 2010; Secher *et al*. 2013; Payros *et al*. 2014), and constitutes a risk factor for the onset of colon tumor genesis (Arthur *et al*. 2012). Colibactin also exacerbates the development of systemic pathologies such as sepsis (Marcq *et al*. 2014) and neonatal meningitis (McCarthy *et al*. 2015). The synthesis of colibactin relies on the *pks* gene cluster (Nougayrède *et al*. 2006) and requires the enzymatic activity of the phosphopantetheinyl transferase ClbA that is encoded on the *pks* island. Recently we showed that the transcription of *clbA* and the production of colibactin were regulated by Fur and RyhB (Tronnet *et al*. 2016). Therefore, iron could constitute a key environmental factor impacting the virulence of *E. coli* strains producing colibactin.

Here, we addressed the question of the role of high iron supply on the production of colibactin. We demonstrate that high iron inhibits the production of colibactin genotoxin

through a complex regulatory mechanism that involves Fur, RhyB and an additional regulator of bacterial iron homeostasis.

Materials and Methods

Bacterial strains, mutagenesis and growth conditions

Bacterial strains used in this study are listed in Table 1. For genetic manipulations, *E. coli* strains were routinely grown at 37°C under shaking in 3ml of Lennox L broth (LB, Invitrogen). Kanamycin (50 µg/ml) or chloramphenicol (25 µg/ml) were added to the medium when required. Inactivation of *E. coli* genes was undertaken using phage λ Red recombinase (Datsenko and Wanner 2000). The primers used to construct mutants are shown in Table 1. Allelic exchanges were confirmed by PCR. For megalocytosis assay, genotoxicity assay and bioluminescence measurements, *E. coli* strains were grown overnight in DMEM containing Hepes (DMEM-Hepes, Gibco, 0.034 µM Fe) supplemented with 100 µM $FeCl_3$ (20.66 µM Fe) when required, at 37°C with shaking. The overnight cultures were then diluted 1:50 in DMEM-Hepes supplemented or not with $FeCl_3$ and grown until OD_{600nm} = 0.6.

Luciferase measurements

Promoter activity of gene *clbA* was determined by time-course quantification of luciferase as previously described (Tronnet *et al*. 2016). *E. coli* was grown overnight in DMEM-Hepes, and subcultured into DMEM-Hepes or DMEM-Hepes supplemented with $FeCl_3$ up to OD_{600nm} = 0.6. Hundred microliter samples were then used to inoculate black 96-well plate (Greiner Bio-one) and grown without shaking at 37°C in a luminometer (Tecan Infinite Pro microplate reader). Both OD_{600nm} and light emission (relative light units, RLU, 6000ms aperture/sample) were recorded every 30 min, simultaneously.

N-myristoyl-D-asparagine quantification by liquid chromatography/mass spectrometry

Quantification of colibactin prodrug motif was performed as previously described (Garcie *et al*. 2016). Briefly, *E. coli* strain M1/5 was grown in DMEM-Hepes with or without $FeCl_3$ at 37°C for 18h. Supernatants of cultures were obtained by centrifugation and filtered. Quantification experiments were conducted with ultra-performance liquid chromatography-high resolution/heated electrospray ionization mass spectrometry (UPLC-HR/HESI-MS). The data were recorded on a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled to a Dionex Ultimate 3000 UPLC. The MS was operated in positive ionization mode at a scan range of 200-500 *m*/*z* and a resolution of 35,000. The spray voltage was set to 3.5 kV, the S-lens to 35, the auxiliary gas heater temperature to 438°C and the capillary temperature to 270°C. Absolute quantification was achieved by using a Schotten-Baumann reaction-derived *N*-myristoyl-L-asparagine (isomer of the *N*-myristoyl-D-asparagine colibactin cleavage product) as a standard. Data were obtained from undiluted cell free sample supernatants, concentrations were calculated using Thermo Xcalibur 2.2 Quan Browser.

Megalocytosis assay

Colibactin induces megalocytosis in cultured eukaryotic cells, manifest as progressive enlargement of the cell body and nucleus and the absence of mitosis. We quantified the extent of colibactin-induced megalocytosis using a methylene blue binding assay (Martin *et al*. 2013). *E. coli* strains were added to HeLa cells at multiplicities of infection (MOIs) of 200 and/or 100, co-cultured for 4h and washed. Cells were then incubated for 72h with cell culture medium containing 200 µg/mL gentamicin followed by staining with methylene blue. Dye binding was determined spectrophotometrically at OD_{660}.

Genotoxicity assay

The capacity of colibactin to engender double strand DNA breaks was determined in HeLa cells by γ-H2AX immunofluorescence analysis (Nougayrède *et al*. 2006); this assay monitors the phosphorylation of histone H2AX, a sensitive marker of ds DNA breaks. HeLa cells (1.5 × 10^4 cells in 200 µl Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 80 µg/mL gentamicin and 0.1 unit/ml bovine insulin) were dispensed into 96-well cell culture plates. After incubation at 37°C in a 5% CO_2 atmosphere for 24h, the cells were washed and incubated with bacteria at MOI = 50 bacteria per cell. After 4h infection at 37°C in 5% CO_2, cells were washed 3 times with Hank's Balanced Salts Solution and incubated at 37°C in cell culture medium for 3h with 200 µg/ml gentamicin. Cells were fixed in the plate with 4% paraformaldehyde and processed as previously described (Martin *et al*. 2013). Rabbit monoclonal anti-γ-H2AX antibody #9718 (Cell Signaling Technology Inc, Danvers MA) was diluted 1:500 in blocking solution and incubated for 2h at room temperature. IRDyeTM 800CW-conjugated goat anti-rabbit secondary antibody was diluted 1:100 in blocking solution and incubated for 1 h. RedDot2 (Biotium) was used for DNA labelling. DNA and γ-H2AX were visualized using an Odyssey Infrared Imaging Scanner (Li-Cor ScienceTec, Les Ulis, France) using 680nm and 800nm channels for RedDot2 and IRDyeTM800 respectively. Relative fluorescence units for γ-H2AX per cell (as determined by γ-H2AX divided by DNA content) were divided by vehicle controls to determine percentage change in phosphorylation of H2AX levels relative to control. All experiments were carried out in triplicate.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 6.0. The mean with standard error of the mean (SEM) 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. 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 ***, ns: not significant.

Results

High iron inhibits the production of colibactin genotoxin by the wild type strain

Recently we showed that the transcription of the *clbA* gene was modulated by iron (Tronnet *et al*. 2016). Therefore, we assessed the impact of iron supplementation on the production of the colibactin. We previously tested a panel of concentrations (1, 10, 100 and 1000 µM) (Tronnet *et al*. 2016). A dose effect of iron was observed on *clbA* expression. We chose to work with 100 µM of $FeCl_3$, because with this concentration the maximum response of *clbA* transcription was reached without inducing toxicity in the bacteria (Tronnet *et al*. 2016).

The production of genotoxin colibactin was measured in wild type (WT) commensal strain M1/5 through bacteria-host cell interaction and subsequent quantification of megalocytosis (Figure 1A) and histone H2AX phosphorylation (Figure 1B) which both correlate with DNA double strand breaks resulting from the genotoxic effect of colibactin (Nougayrède *et al*. 2006). This revealed that the supplementation of the interaction medium (DMEM-Hepes) with $FeCl_3$ abrogated the genotoxic effect induced by the colibactin (Figure 1A, 1B).

The mature colibactin genotoxin has not been fully characterized yet. However, a biosynthetic by-product derived from the colibactin assembly line, *i.e.* the N-myristoyl-D-asparagine moiety, has been recently characterized (Bian *et al*. 2013; Brotherton and Balskus 2013; Vizcaino and Crawford 2015). This moiety is a prodrug motif that is cleaved during the late activation step (Dubois *et al*. 2011). We developed a LC-MS assay to quantitatively measure the amount of N-myristoyl-D-asparagine as a means to indirectly quantify the production of

the genotoxin (Garcie *et al*. 2016) by *E. coli* M1/5 (Figure 1C). The results showed that in high iron conditions, the quantity of colibactin prodrug motif was abolished in the bacterial culture supernatant compared to the DMEM-Hepes (Figure 1C).

Altogether, these data showed that high iron inhibited the synthesis of colibactin in a wild type strain.

High iron inhibits both the transcription of clbA and the production of colibactin in Δfur mutant strains

Recently we showed that the transcription of the *clbA* gene and the production of colibactin was regulated by Fur. Therefore, we assessed the impact of high iron on the Fur-dependent regulation of *clbA* and colibactin.

E. coli strain Nissle 1917 harboring a transcriptional luciferase fusion with the *clbA* gene and its Δ*fur* isogenic mutant were analyzed (Table 1, Homburg *et al*. 2007; Tronnet *et al*. 2016). Both strain Nissle *clbA-lux* and strain Nissle *clbA-lux* Δ*fur* were grown in chemically defined DMEM-Hepes and DMEM-Hepes supplemented with $FeCl_3$ (100µM). The transcription rate of *clbA* was determined as OD_{600nm} standardized-relative luminescence units (RLU) (Figure 2A). An increase of bioluminescence emission was observed during the bacterial growth, to reach a maximal value at early stationary phase of growth, followed by a decrease of transcription, for both strains (Figure 2A). In iron-supplemented conditions, the *clbA* transcription was strongly repressed in both strain Nissle *clbA-lux* and strain Nissle *clbA-lux* Δ*fur* (Figure 2A).

The production of colibactin was measured in *E. coli* WT strains and Δ*fur* mutant derivatives through bacteria-host cell interaction and subsequent quantification of megalocytosis in the absence and presence of iron supplementation ($FeCl_3$, 100µM, Figure 2B). Two distinct genetic contexts of *E. coli* were investigated: Nissle 1917 (probiotic strain) and M1/5

(commensal strain). This revealed that in high iron interaction medium, the genotoxic effect induced by the colibactin was significantly diminished both in the WTs and the Δ*fur* inactivated mutants.

Altogether, these data suggested that in Δ*fur* mutants the iron-dependent *clbA* repression and the resulting decreased colibactin production could occur either through RyhB in a Fur-independent manner or through an additional regulator.

High iron inhibits both the transcription of clbA and the production of colibactin in Δryhb mutant strains

Recently we showed that the transcription of the *clbA* gene and the production of colibactin was regulated by RyhB. Therefore, we assessed the impact of high iron on the RyhB-dependent regulation of *clbA* and colibactin.

E. coli strain Nissle *clbA-lux* and its Δ*ryhB* isogenic mutant were analyzed (Table 1, Homburg *et al*. 2007; Tronnet *et al*. 2016). Both strain Nissle *clbA-lux* and strain Nissle *clbA-lux* Δ*ryhB* were grown in DMEM-Hepes and DMEM-Hepes supplemented with $FeCl_3$ (100µM; Figure 3A). This revealed that in iron-supplemented conditions, the *clbA* transcription was repressed in both strains Nissle *clbA-lux* and Nissle *clbA-lux* Δ*ryhB* (Figure 3A).

The production of colibactin was measured in *E. coli* WT strains and their Δ*ryhB* mutant derivatives through bacteria-host cell interaction and subsequent quantification of megalocytosis in the absence and presence of iron supplementation ($FeCl_3$, 100µM, Figure 3B). This revealed that in high iron interaction medium, the genotoxic effect induced by the colibactin was significantly diminished both in the WTs and the Δ*ryhB* inactivated mutants.

Altogether, these data suggested that in a Δ*ryhB* mutant the iron-dependent *clbA* repression and the resulting decreased colibactin production could occur through Fur in a *ryhB*-independent manner, or through an additional regulator.

A yet unknown factor is involved in the high iron-dependent regulation of colibactin production

To address the question of the potential involvement of an additional regulatory factor in the regulation of colibactin, Δ*ryhB* Δ*fur* double mutants were constructed in strains Nissle 1917 and M1/5, and were analyzed for their capacity to produce colibactin by quantification of megalocytosis (Figure 4A). This revealed that the quantity of colibactin produced in the double mutants was highly decreased, compared to the WT strains. The genotoxic effect measured in the double mutants was lower than that observed for the Δ*fur* inactivated mutants, in each genetic context (Figure 4A).

To test the putative involvement of an additional regulatory factor in the high iron-dependent control of colibactin production, megalocytosis assay was performed with the double mutants Δ*ryhB* Δ*fur* in the presence of high iron (100µM, Figure 4B). This revealed that the colibactin-associated genotoxic effect was diminished by iron supplementation in the Δ*ryhB* Δ*fur* mutants.

Altogether, these findings suggested that an additional yet unknown regulatory factor was involved in the regulation of the colibactin in response to high iron conditions.

Discussion

Our previous study (Tronnet *et al*. 2016) highlighted that the mutation of *ryhB* led to an increase of colibactin production, whereas the mutation of *fur* resulted in a decrease of colibactin production in three different genetic contexts of *E. coli* belonging to the B2 phylogenetic group. Here we showed that high iron decreases *clbA* transcription and colibactin production in wild type, Δ*ryhB*, Δ*fur* and Δ*ryhB* Δ*fur* strains. This suggests an iron-dependent synthesis of the genotoxin through a non-classic Fur/RyhB-mediated pathway. Nowadays, numerous reports

support the four modes of Fur regulation, apo- and holo-Fur activation and repression, establishing a significant deviation from the classical model of Fur regulation (Butcher *et al*. 2012). In the same manner, *ryhB* was reported to be regulated in a Fur-dependent and Fur-independent manner, and to repress or activate genes (Massé *et al*. 2005; Papenfort and Vanderpool 2015).

Colibactin is a virulence factor involved in neonatal systemic infections (McCarthy *et al*. 2015), and is also associated with the development of colorectal cancer (Arthur *et al*. 2012; Cougnoux *et al*. 2014; Dalmasso *et al*. 2014). Our work could make a link between iron concentration in the gut/the blood and *E. coli*-mediated carcinogenesis/systemic infections. Given the existence of a gradient of iron concentration from the lumen to the intestinal epithelial cell, it is conceivable that the fine-tuning of *clbA* expression allows the production of colibactin when the pathogenic *E. coli* is located in an appropriate site in the gut. This iron-dependent regulation could also be a reason for the emergence of B2 group *E. coli*, as we know there is a crosstalk between colibactin and siderophores (Martin *et al*. 2013).

Integrating the regulation of virulence factors, such as siderophores and colibactin, into networks that respond to specific environmental signals, such as the local iron concentration and the balance between Fur and RyhB could result in an accurate production of colibactin and siderophores, so that the bacteria can adapt to the competitive environment that is the gut, or in the blood.

This work highlights the complex regulatory mechanism of an important bacterial virulence and carcinogenesis factor by major regulators of bacterial iron homeostasis, and provides insights into the consequences associated with high iron supply (graphical abstract). Further understanding of how *E. coli* senses environments to regulate the biosynthesis of colibactin may uncover novel pathways for the development of potential targets against this pathogenic enterobacteria.

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Conflict of interest

The authors have no conflict of interest to declare.

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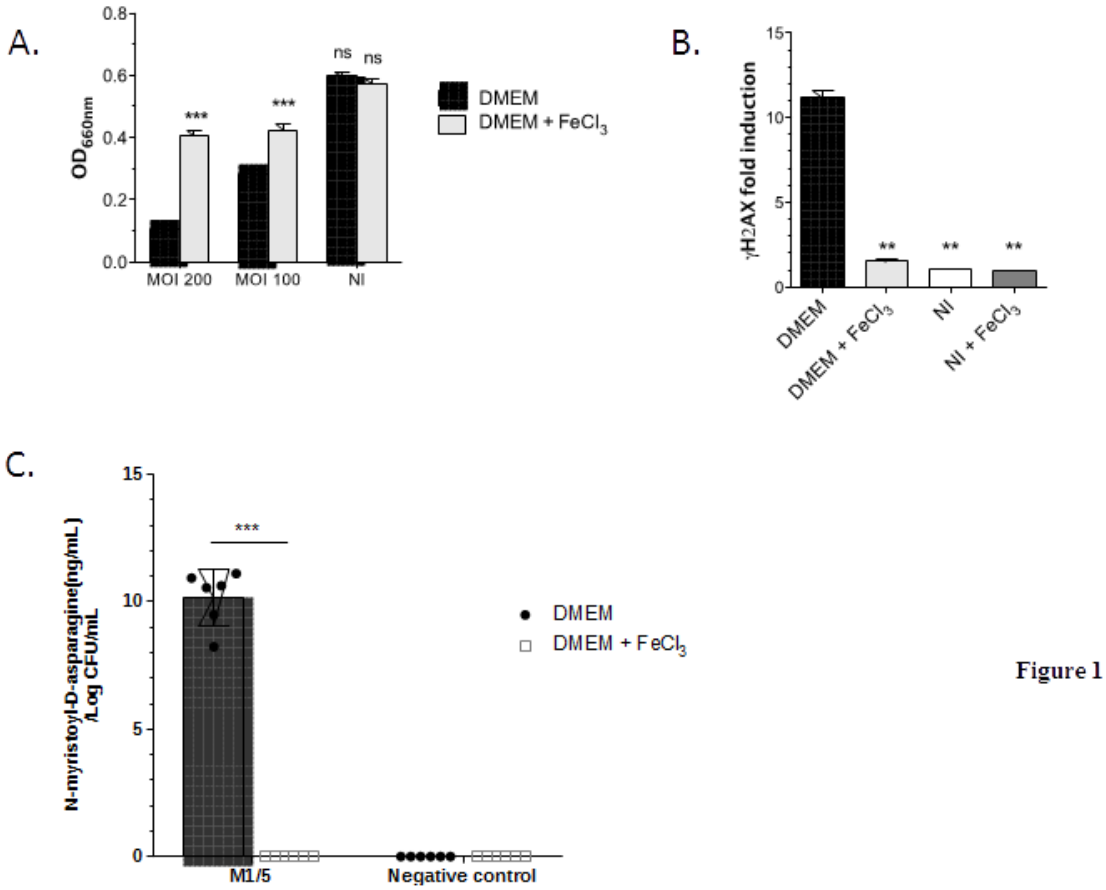

Figure 1

Figure 1. High iron inhibits the production of colibactin genotoxin by the wild type strain.

A. The production of colibactin by *E. coli* strain M1/5 was determined by quantification of megalocytosis as previously described (Martin *et al.* 2013). HeLa cells were infected with *E. coli*, in the presence of 100µM $FeCl_3$ or not, then washed 4h post infection. After infection, the cells were incubated for 72h with gentamicin before protein staining with methylene blue. The staining was quantified by acid-extraction of the methylene blue and measurement of optical density at 660nm ($OD_{660\ nm}$). Multiplicity of infection (number of bacteria per cells): MOI = 200 and 100. **B**. The production of colibactin by *E. coli* strain M1/5 was determined by quantification of H2AX phosphorylation. HeLa cells were infected with *E. coli*, in the presence of 100µM $FeCl_3$ or not, then washed 4h post infection. The cells were then incubated 3h in DMEM medium supplemented with gentamicin before fixation, then

permeabilized and labeled for DNA and phosphorylated H2AX histone (γ-H2AX) using an In-Cell Western method (Martin *et al*. 2013). MOI = 50. **C.** The colibactin prodrug motif *N*-myristoyl-D-asparagine produced by *E. coli* strain M1/5 was quantified by LC-MS. Bacteria were cultivated at 37°C for 18h in DMEM-Hepes or DMEM-Hepes supplemented with $FeCl_3$ (100µM), and *N*-myristoyl-D-asparagine was quantified in culture supernatants and pellets by LC-MS using *N*-myristoyl-l-asparagine (isomer of the *N*-myristoyl-D-asparagine colibactin prodrug motif) as a standard. The results were normalized to the bacterial biomass and are presented as quantity of *N*-myristoyl-D-asparagine (ng/ml). Data represented in the graph were obtained from three biological replicates and two independent experiments. NI: not infected. Statistical analysis: unpaired t-test. ***: $p < 0.001$, **: $p < 0.01$. ns: not significant.

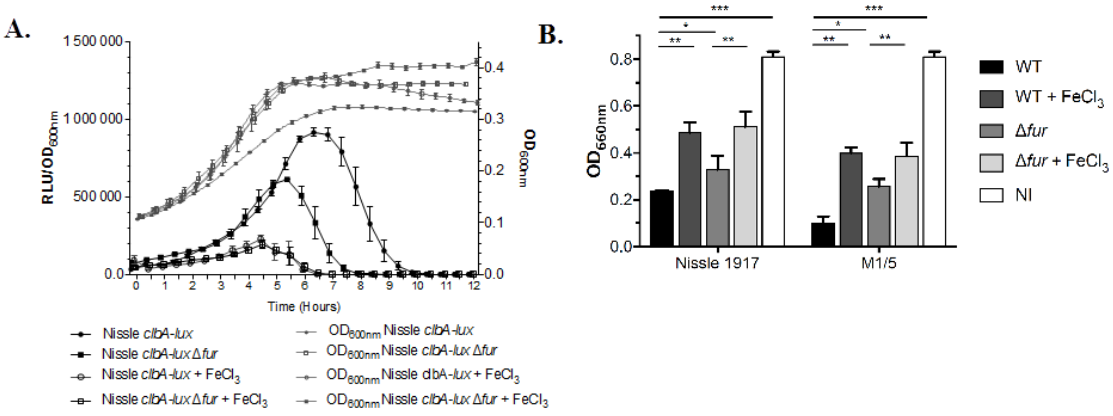

Figure 2..

Figure 2. High iron inhibits the transcription of *clbA* and the production of colibactin genotoxin in Δ*fur* mutant strains

A. Growth curves (OD_{600nm}) and relative OD_{600nm} standardized-relative luminescence units (RLU/OD_{600nm}) of strains Nissle *clbA-lux* and Nissle *clbA-lux* Δ*fur* grown at 37°C in DMEM-Hepes or DMEM-Hepes supplemented with 100 µM of $FeCl_3$. The given mean RLU average

values with SEM result from four independent experiments. **B.** The production of colibactin by *E. coli* strains Nissle 1917 and M1/5 (wild type and Δ*fur*) was determined by quantification of megalocytosis, in the absence and the presence of $FeCl_3$ (100 µM). MOI = 200. The given quantification average values are represented as mean values and SEM and result from three independent experiments. Statistical analysis was performed using Two-way ANOVA and Bonferroni post-test compared to mean values obtained for wild type strains. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. NI: not infected.

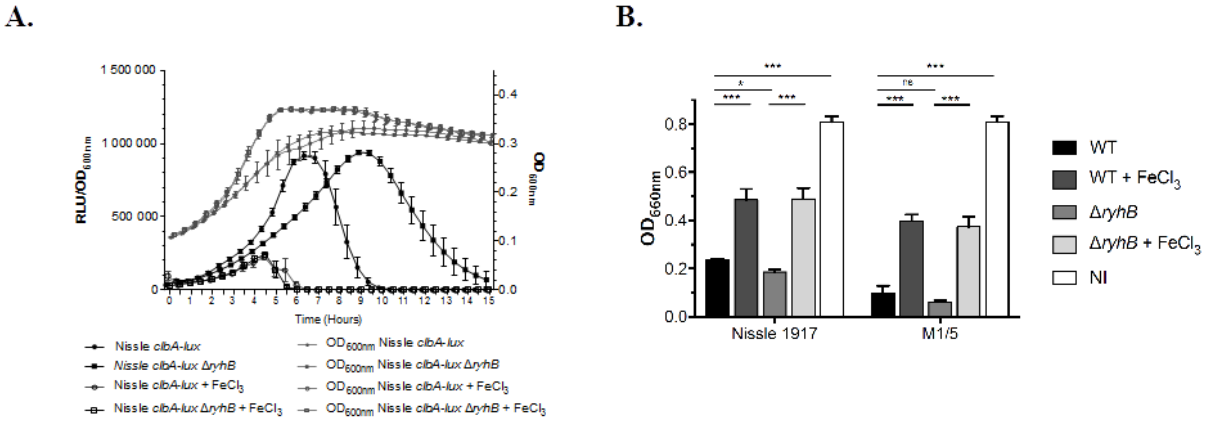

Figure 3.

Figure 3. High iron inhibits the transcription of *clbA* and the production of colibactin genotoxin in Δ*ryhB* mutant strains

A. Growth curves (OD_{600nm}) and relative OD_{600nm} standardized-relative luminescence units (RLU/OD_{600nm}) of strains Nissle *clbA-lux* and Nissle *clbA-lux* Δ*ryhB* grown at 37°C in DMEM-Hepes or DMEM-Hepes supplemented with 100 µM of $FeCl_3$. The given mean RLU average values with SEM result from four independent experiments. **B.** The production of colibactin by

E. coli strains Nissle 1917 and M1/5 (wild type and Δ*ryhB*) was determined by quantification of megalocytosis, in the absence and the presence of $FeCl_3$ (100 µM). MOI = 200. The given quantification average values are represented as mean values and SEM and result from three independent experiments. Statistical analysis was performed using Two-way ANOVA and Bonferroni post-test compared to mean values obtained for wild type strains. ***$P < 0.001$; *$P < 0.05$; ns: not significant. NI: not infected.

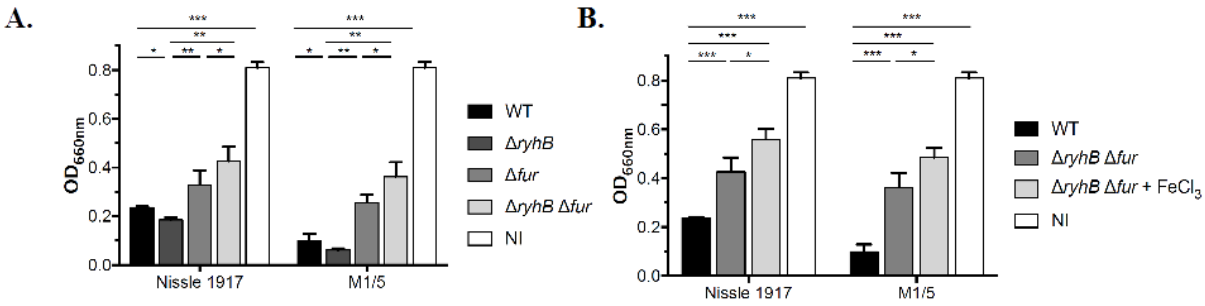

Figure 4.

Figure 4. A yet unknown factor is involved in the high iron-dependent regulation of colibactin production

A. The production of colibactin by *E. coli* strains Nissle 1917 and M1/5 (wild type, Δ*ryhB*, Δ*fur* and Δ*ryhB* Δ*fur*) was determined by quantification of megalocytosis. MOI = 200. The given quantification average values are represented as mean values and SEM and result from three independent experiments. Statistical analysis was performed using Two-way ANOVA and Bonferroni post-test compared to mean values obtained for wild type strains. **B.** The production of colibactin by *E. coli* strains and Nissle 1917 and M1/5 (wild type and Δ*ryhB* Δ*fur*) was determined by quantification of megalocytosis. The infection of HeLa cells with *E. coli* was

performed in the absence or the presence of $FeCl_3$ (100 µM). MOI = 200. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. NI: not infected.

Table 1. Strains and primers used in this study.

***E. coli* strain**		**Source or reference**
Nissle *clbA-lux*	Luciferase fusion of the gene *clbA*, Kan^R	Homburg *et al*. 2007
Nissle *clbA-lux* Δ*ryhB*	*ryhB* mutant of strain Nissle *clbA-lux*, $Kan^R Cm^R$	Tronnet *et al*. 2016
Nissle *clbA-lux* Δ*fur*	*fur* mutant of strain Nissle *clbA-lux*, $Kan^R Cm^R$	Tronnet *et al*. 2016
Nissle *clbA-lux* Δ*ryhB* Δ*fur*	*ryhB fur* double mutant of strain Nissle *clbA-lux* $Kan^R Cm^R$	This study
M1/5	Colibactin producer, commensal strain	Martin *et al*. 2013
M1/5 Δ*ryhB*	*ryhB* mutant of strain M1/5, Cm^R	Tronnet *et al*. 2016
M1/5 Δ*fur*	*fur* mutant of strain M1/5, Kan^R	Tronnet *et al*. 2016
M1/5 Δ*ryhB* Δ*fur*	*ryhB fur* double mutant of strain M1/5, Cm^R	This study
Nissle 1917	Colibactin producer, probiotic strain	Olier *et al*. 2012
Nissle 1917 Δ*ryhB*	*ryhB* mutant of strain Nissle 1917, Cm^R	Tronnet *et al*. 2016
Nissle 1917 Δ*fur*	*fur* mutant of strain Nissle 1917, Kan^R	Tronnet *et al*. 2016
Nissle 1917 Δ*ryhB* Δ*fur*	*ryhB fur* double mutant of strain Nissle 1917, $Cm^R Amp^R$	This study
Primers		
Fur_F	CGCCCTAAAGAAAGCTGGCC	Tronnet *et al*. 2016
Fur_R	CCTTCGTGCGCATGTTCATC	Tronnet *et al*. 2016
CMD1171_ST (*ryhB:cat*)	TTTGGGGTAAATGTCCCTTTC	Tronnet *et al*. 2016
CMD1172_ST *(ryhB:cat)*	GTGCGCATAACGAACACAAG	Tronnet *et al*. 2016