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Iron Homeostasis Regulates the Genotoxicity of *Escherichia coli* That Produces Colibactin

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The genotoxin colibactin is a secondary metabolite produced by a variety of pathogenic enterobacteria. Its biosynthesis requires the enzymatic activity of the phosphopantetheinyl transferase (PPTase) ClbA. We previously showed that ClbA can also contribute to the production of siderophores. Because the biosynthesis of siderophores is regulated by iron availability, we hypothesized that iron could also modulate the production of colibactin through the transcriptional regulation of *clbA*. This study revealed an increased transcription of *clbA* under iron-limiting conditions and a decrease of *clbA* expression in iron-rich media. We demonstrate that *clbA* transcription is regulated by both the ferric uptake regulator (Fur) and the small regulatory noncoding RNA RyhB. We evidenced that the regulation of the transcription of *clbA* by Fur and RyhB leads to the regulation of colibactin production. This work highlights the complex mechanism of regulation of an important virulence factor by the two major regulators of bacterial iron homeostasis, making iron a key environmental factor contributing to bacterial virulence and carcinogenesis.

In the human body, most of the iron is intracellular or sequestered by proteins, which makes it unavailable to invading microbes. This process, i.e., nutritional immunity, is a critical host defense strategy against bacterial pathogens that perceive the scarcity of iron within the vertebrate host as a means to sense that they are in their host (1). The global response to iron deprivation in bacteria relies on the ferric uptake regulator (Fur) protein and the small regulatory noncoding RNA (sRNA) RyhB (2–4). In the classical Fur regulation paradigm, Fur binds ferrous ions and the dimeric Fe^{2+}-Fur complex (holo-Fur) recognizes target sequences upstream of iron-regulated genes and represses their transcription. However, nowadays numerous reports support four modes of Fur regulation, i.e., apo- and holo-Fur activation and repression, establishing a significant deviation from the classical model of Fur regulation (5). In bacteria, archaea, and eukaryotes, small riboregulators have been shown to mediate posttranscriptional mechanisms of gene regulation (6). In many cases, sRNAs form base pairs with and sequester mRNA ribosome-binding sites, resulting in translational repression and accelerated transcript decay. In contrast, a growing number of examples of translational activation and mRNA stabilization by sRNAs have now been documented. A given sRNA often employs a conserved region to interact with and regulate both repressed and activated targets (6, 7).

To counteract iron deprivation, bacterial pathogens synthesize small iron-scavenging molecules, i.e., siderophores, that are crucial for their survival and play a significant role in virulence (8–10). Siderophores are nonribosomal peptides (NRP) or polyketide (PK)-NRP hybrids (11). A prerequisite for the synthesis of all NRPs, PKs, and PK-NRP hybrids is the posttranslational attachment of P-Pant arms from coenzyme A (CoA) to a conserved serine residue of a carrier protein, converting inactive apo-synthases to active holo-synthases. This reaction is catalyzed by members of the type II family of phosphopantetheinyl transferases (PPTases) (12). The core genome of *Escherichia coli* codes for two type II PPTases, i.e., EntD and YieE (*ECK3705*, *b3712*, *JW3690*) (13). Whereas the role of YieE remains elusive, EntD is the PPTase involved in the biosynthesis of the siderophores enterobactin, salmochelin, and yersiniabactin.

An additional type II PPTase, i.e., ClbA, is encoded on the *pks* gene cluster, responsible for the synthesis of the PK-NRP hybrid colibactin in diverse pathogenic enterobacteria, including approximately 50% of the *E. coli* strains that belong to phylogenetic group B2 (14). Colibactin is a genotoxin that induces DNA double-strand breaks, senescence, and chromosomal abnormalities in eukaryotic cells (15–17) and was demonstrated to be a bona fide virulence factor in a mouse model of sepsis (18) and in a rat model of neonatal meningitis (19). We have recently highlighted the existence of cross talk between colibactin and siderophore biosynthesis (20). Indeed, ClbA can replace EntD and contribute to siderophore biosynthesis. Therefore, we speculated that this interplay could constitute a fitness advantage for *E. coli* and that iron could play a key role in this connection.

Here we show that the expression of *clbA* is controlled by iron bioavailability and that the production of colibactin is regulated by Fur and RyhB. Therefore, iron could constitute a key environmental factor contributing to the virulence and carcinogenicity of *E. coli* strains producing colibactin.

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TABLE 1 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence[a]	Reference or source
E. coli strains		
Nissle 1917 *clbA-lux*	Luciferase fusion of the gene *clbA*, Kan^r	22
Nissle 1917 *clbR-lux*	Luciferase fusion of the gene *clbR*, Kan^r	22
Nissle 1917 *clbB-lux*	Luciferase fusion of the gene *clbB*, Kan^r	22
Nissle 1917 *clbQ-lux*	Luciferase fusion of the gene *clbQ*, Kan^r	22
Nissle 1917 *clbA-lux* Δ*ryhB*	*ryhB* mutant strain, Kan^r Cm^r	This study
Nissle 1917 *clbA-lux* Δ*ryhB*+p-*ryhB*	*ryhB* mutant strain carrying p-*ryhB*, Kan^r Cm^r Amp^r	This study
Nissle 1917 *clbA-lux* Δ*fur*	*fur* mutant strain, Kan^r Cm^r	This study
Nissle 1917 *clbA-lux* Δ*fur*+p-*fur*	*fur* mutant strain carrying p-*fur*, Kan^r Cm^r Amp^r	This study
M1/5	Colibactin producer, commensal strain	20
M1/5 Δ*ryhB*	*ryhB* mutant strain, Cm^r	This study
M1/5 Δ*ryhB*+p-*ryhB*	*ryhB* mutant strain carrying p-*ryhB*, Cm^r Amp^r	This study
M1/5 Δ*fur*	*fur* mutant strain, Kan^r	This study
M1/5 Δ*fur*+p-*fur*	*fur* mutant strain carrying p-*fur*, Kan^r Amp^r	This study
Nissle 1917	Colibactin producer, probiotic strain	16
Nissle 1917 Δ*clbA*+pBAD33-*clbA*		This study
Nissle 1917 Δ*clbA*+pBAD33-*clbA*	*clbA* mutant strain carrying pBAD33-*clbA*, Kan^r	This study
Nissle 1917 Δ*clbA*+pBAD33-*clbA*Δ*clbR*	*clbA* mutant strain carrying pBAD33-*clbA*Δ*clbR*, Kan^r	This study
Nissle 1917 Δ*ryhB*	*ryhB* mutant strain, Cm^r	This study
Nissle 1917 Δ*ryhB*+p-*ryhB*	*ryhB* mutant strain carrying p-*ryhB*, Cm^r Amp^r	This study
Nissle 1917 Δ*fur*	*fur* mutant strain, Kan^r	This study
Nissle 1917 Δ*fur*+p-*fur*	*fur* mutant strain carrying p-*fur*, Kan^r Amp^r	This study
SP15	Colibactin producer, pathogenic strain	35
SP15 Δ*ryhB*	*ryhB* mutant strain, Cm^r	This study
SP15 Δ*ryhB*+p-*ryhB*	*ryhB* mutant strain carrying p-*ryhB*, Cm^r Amp^r	This study
SP15 Δ*fur*	*fur* mutant strain, Kan^r	This study
SP15 Δ*fur*+p-*fur*	*fur* mutant strain carrying p-*fur*, Kan^r Amp^r	This study
MG1655 Δ*ryhB*	*ryhB* mutant strain, Cm^r	Gift from C. M. Dozois
Bw25113 Δ*fur*	*fur* mutant strain, Kan^r	Gift from C. M. Dozois
Plasmids		
p-*ryhB*	High-copy-no. pSC-A plasmid carrying the *ryhB* gene; Amp^r Kan^r	This study
p-*fur*	High-copy-no. pSC-A plasmid carrying the *fur* gene; Amp^r Kan^r	This study
pBAD33-T7-*Venus*	High-copy-no. pBAD33 plasmid containing the *Venus* gene coding for a variant of the yellow fluorescent protein of *Aequorea victoria*	This study
pBAD33-T7-*clbA*	High-copy-no. pBAD33-T7 plasmid carrying the *Venus* fusion of the *clbA* gene	This study
pBAD33-T7-*clbA*Δ*clbR*	High-copy-no. pBAD33-T7 plasmid carrying the *Venus* fusion of the *clbA* gene and mutated *clbR*	This study
Primers		
Fur-P1	AAAATCCTGGAAGTTCTTCAGGAGCCGGACAACCATCACGTCAGTGCGGAGTGTAGG CTGGAGCTGCTTC	This study
Fur-P2	TTCGCGGCAATCGCCTTCGGCACAGTGACCGTAAAGATAGAGACTGTGGTCATATGA ATATCCTCCTTAG	This study
Fur_F	CGCCCTAAAGAAAGCTGGCC	This study
Fur_R	CCTTCGTGCGCATGTTCATC	This study
Fur_F_Compl	CTGTAAGCTGTGCCACGTTTT	This study
Fur_R _compl	CTGAGAGCTGTAACTCTCGCTTTTC	This study
CMD1171_ST (*ryhB*::*cat*)	TTTGGGGTAAATGTCCCTTTC	29
CMD1172_ST (*ryhB*::*cat*)	GTGCGCATAACGAACACAAG	29
RyhB_F_Compl	CCTCTCGAGAAAGCGGACGTGGTTCCTAC	This study
RyhB_R_Compl	GTACTCGAGTGTTTCTGCGTGGCGTATTAC	29
Venus-XbaI	GCGCTCTAGAATGGTGTCTATCACTAAAGATCAAATC	This study
Venus-fus-as	GCGCGTCGACTTACTTGTACAGCTCGTCCATG	This study
T7-term-fw	AAGGGGATCCGGCTGCTAAC	This study
T7-term-rev	GCGCAATTGCGGATATAGTTCCTCCTTTCAG	This study
pBAD33-*Venus*-Bam-rev	GCGCGCGGATCC*C*GGCTGCTAACAAAG	This study
pBAD33-*Venus*-fw	P-TGGTGTCTATCACTAAAGATCAAATC	This study
*clbA*p-BamHI-fw	GCGCGGATCCAACCATCACCTTATTATCGG	This study
*clbA*p-rev1-1	TTAGATAATCTCATTCCTGTTAGC	This study

(Continued on following page)

TABLE 1 (Continued)

Strain, plasmid, or primer	Description or sequence[a]	Reference or source
*clbA*p-Del*clbR*-fw2	CCGTTATCTAAGACAAGTATTGCGCATG	This study
*clbA*p-Del*clbR*-rev2	ACTTGTCTTAGATAACGGGTTTTTTTCTTTG	This study
fyuA-up	CAACGCGCAGGCCTTTAC	This study
fyuA-rev	GCGTGCTTTCGTCTTGCTG	This study
clbC-up	CGAGTCAAATGCGCCATCAC	This study
clbC-rev	GGACCGCCATACCAATAATG	This study
JPN41	CAGATACACAGATACCATTC	14
JPN42	TCAATGAGGAAGAAATAAAAC	14

[a] Restriction sites are underlined. A P in front of the sequence indicates a phosphate residue.

MATERIALS AND METHODS

Bacterial strains, mutagenesis, and growth conditions. The bacterial strains used in this study are listed in Table 1. For genetic manipulations, *E. coli* strains were routinely grown at 37°C with shaking at 240 rpm in 3 ml of Lennox L broth (LB; Invitrogen). Ampicillin (50 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (25 μg/ml) was added to the medium when required. Gene inactivations were performed using the bacteriophage lambda Red recombinase method (21) and the primers listed in Table 1. Briefly, the *ryhB*::*cat* and *fur*::*kan* alleles were PCR amplified from the chromosomal DNA of strain MG1655 Δ*ryhB* or Bw25113 Δ*fur*, respectively, using primers CMD1171_ST/CMD1172_ST or Fur_F/Fur_R, respectively. The purified PCR product was used to transform the strains of interest. The allelic exchanges were confirmed by PCR using the same pairs of primers. The resulting mutants where *ryhB* or *fur* was disrupted by a resistance cassette were named the Δ*ryhB* and Δ*fur* mutants, respectively. To generate the *fur*::*cat* mutation in strain Nissle 1917 *clbA-lux*, the chloramphenicol cassette was PCR amplified using primers Fur_P1/Fur_P2. The purified PCR product was used to transform *E. coli* Nissle 1917 *clbA-lux*. The transformants were tested by PCR amplification using primers Fur_F/Fur_R. For cloning of the wild-type *ryhB* and *fur* genes in cloning vectors, the *ryhB* and *fur* genes were PCR amplified using primer pairs RyhB_F_Compl/RyhB_R_Compl and Fur_F_Compl/Fur_R_Compl, respectively. The resulting PCR products were then cloned into the pSC-A vector using a StrataClone PCR cloning kit (Agilent Technologies). The presence of the appropriate insert in the resulting plasmids was checked by PCR amplification, and the plasmids were transformed into the mutant strains.

For the megalocytosis assay and assays for bioluminescence and fluorescence measurements, the *E. coli* strains were grown overnight in Dulbecco's modified Eagle's medium (DMEM) containing HEPES (DMEM-HEPES; Gibco), supplemented with antibiotics when required, at 37°C with shaking (240 rpm). The overnight cultures were then diluted 1:50 in DMEM-HEPES supplemented or not with iron ($FeCl_3$, 100 μM; $FeSO_4$, 100 μM), magnesium ($MgSO_4$, 100 μM), copper ($CuCl_2$, 100 μM), or iron chelator (8-hydroxyquinoline [8-HQ; 40 μM] or desferrioxamine [DFO; 0.2 μM]) and grown until the optical density at 600 nm (OD_{600}) was 0.6. The ranges of concentrations of the chelator and the iron supplement were analyzed, and the highest concentrations of substances which did not alter bacterial growth were chosen.

Construction of *Venus* fusions. A low-copy-number vector system was used to generate *clbA* promoter fusions with the *Venus* gene as a reporter. For this purpose, a derivative of vector pBAD33, which was devoid of the elements required for L-arabinose-dependent expression, was employed. An overview about the construction of the plasmids is given in Fig. S3 in the supplemental material.

In more detail, the translational fusion of the first 24 bp of *rplL* and the *Venus* gene, coding for a variant of the yellow fluorescent protein of *Aequorea victoria*, was amplified from pMB*rplL-Venus* using the primers Venus-XbaI and Venus-fus-as. The PCR product was digested with XbaI and SalI and cloned into pBAD33 (see step 1 in Fig. S3 in the supplemental material). The resulting plasmid was digested with EcoRV and SacI, treated with mung bean nuclease (MBN), and religated as plasmid pBAD33-*Venus* in order to delete the *araBAD* promoter and the *araC* gene (see step 2 in Fig. S3). Since the aim was to clone the whole *clbBR* intergenic region together with the *clbR* gene, which harbors the *clbA* promoter, the transcription into the opposite direction of *clbA*, facing *clbB*, had to be blocked. To achieve this, the T7 terminator sequence of pTXB1 (NEB) was amplified with primers T7-term-for and T7-term-rev, subsequently digested with BamHI and MfeI, and cloned upstream of *Venus* into pBAD33-*Venus*, resulting in pBAD33-T7-*Venus* (see step 3 in Fig. S3). The plasmid was verified by sequencing.

The whole pBAD33-T7-*Venus* sequence was amplified via PCR as a precursor for the following plasmids by using primers pBAD33-*Venus*-Bam-rev and pBAD33-*Venus*-fw and subsequently digested with BamHI (see step 4 in Fig. S3). To yield pBAD33-T7-*clbA*, the 648-bp DNA sequence upstream of *clbA* in *E. coli* strain M1/5 (20), comprising the putative *clbA* promoter, the *clbR* gene, as well as the whole *clbBR* intergenic region (*clbBR* in. reg.), was amplified with primers *clbA*p-BamHI-fw and *clbA*p-rev1-1, digested with BamHI, and then ligated with the digested pBAD33-T7-*Venus* amplicon (see step 5a in Fig. S3). To generate pBAD33-T7-*clbA*Δ*clbR*, a derivative of pBAD33-T7-*clbA* with a deletion of *clbR* nucleotides 33 to 40, a two-step-PCR was carried out using *E. coli* M1/5 as a template (see step 5b in Fig. S3). Amplicons were generated with the primer pairs *clbA*p-BamHI-fw/*clbA*p-Del*clbR*-rev2 (PCR 1) and *clbA*p-Del*clbR*-fw2/*clbA*p-rev1-1 (PCR 2). PCR products 1 and 2 were fused using primers *clbA*p-BamHI-fw and *clbA*p-rev1-1 (PCR 3) and then digested with BamHI and ligated with PCR-amplified and BamHI-digested pBAD33-T7-*Venus*, resulting in pBAD33-T7-*clbA*Δ*clbR*. The plasmids were verified by sequencing.

Luciferase and Venus measurements. The promoter activities of genes *clbA*, *clbB*, *clbQ*, and *clbR* in different media were determined by time course quantification of luciferase or Venus expression. When an OD_{600} of 0.6 was reached, bacterial subcultures were diluted to an OD_{600} of 0.1 in the appropriate medium. Samples of 100 μl were then used to inoculate a black 96-well plate (Greiner Bio-One), and the bacteria were grown without shaking at 37°C in a luminometer (Tecan Infinite Pro microplate reader). For luminescence measurements, light emission (relative light units [RLU]) was recorded (6,000-ms aperture per sample) every 30 min in parallel with the OD_{600}. For Venus fluorescence measurements, fluorescence was recorded at 535 nm (bandwidth, 25 nm) using an excitation wavelength of 485 nm (bandwidth, 20 nm) with the luminometer. The OD_{600} was measured in parallel at each time point. The area under the curve (AUC), which quantifies the cumulative luminescence or fluorescence, was calculated with GraphPad Prism (version 6.0) software.

Quantification of colibactin-associated genotoxic effect by megalocytosis assay. Quantification of the colibactin-associated genotoxic effect by megalocytosis assay was performed as previously described (14). Briefly, HeLa cells were grown in DMEM (GlutaMAX; Invitrogen) supplemented with 10% (vol/vol) fetal calf serum (FCS; Eurobio) and 1%

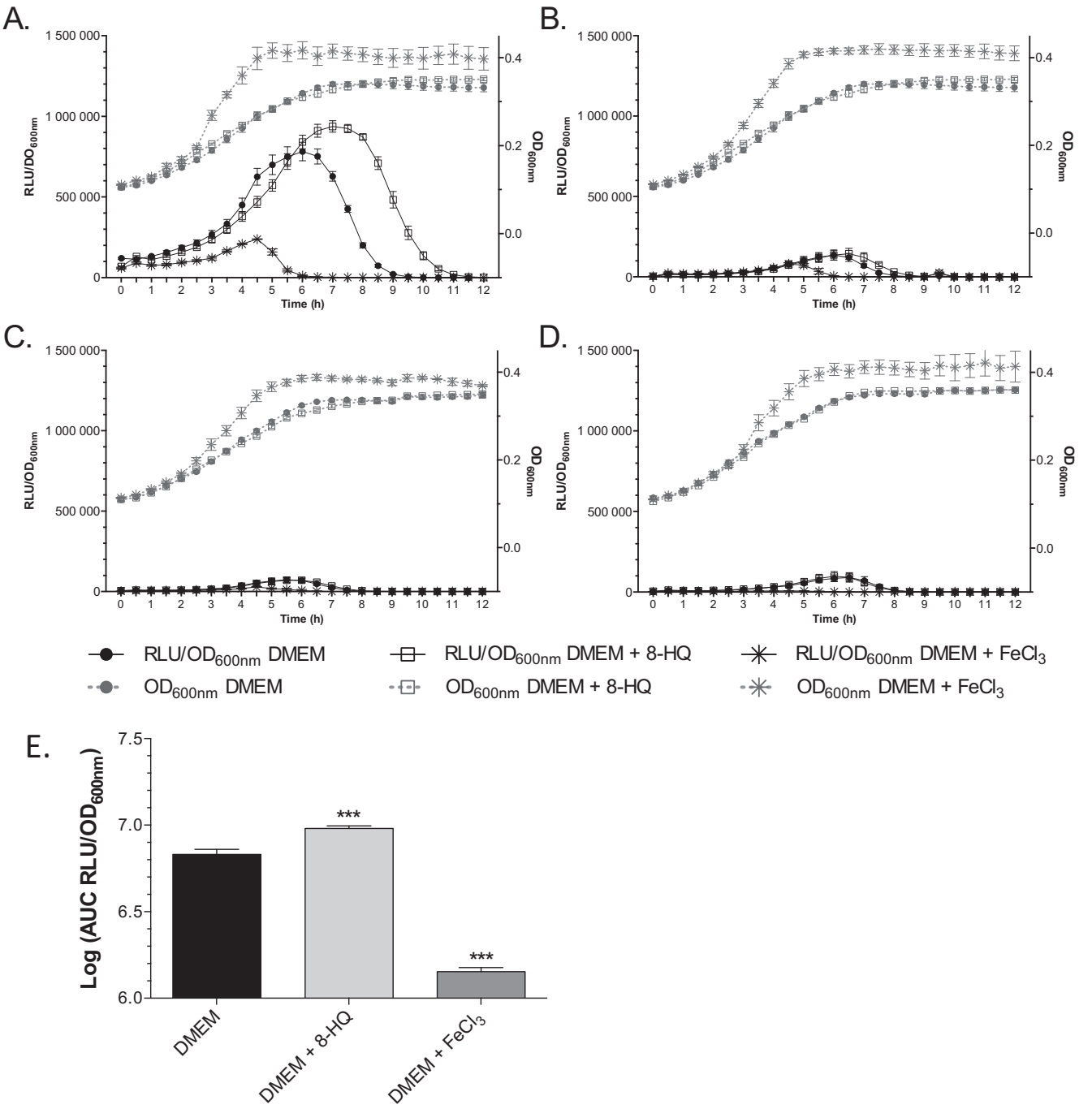

FIG 1 Iron availability modulates the transcription of *clbA*. Growth curves (OD_{600}) and the RLU/OD_{600} of Nissle 1917 *clbA-lux* (A), *clbB-lux* (B), *clbQ-lux* (C), and *clbR-lux* (D) fusion strains grown at 37°C in DMEM-HEPES, DMEM-HEPES supplemented with 40 μM 8-HQ, or DMEM-HEPES supplemented with 100 μM $FeCl_3$. (E) AUC, determined with GraphPad Prism (version 6.0) software, of the RLU/OD_{600} of *clbA* under the three different conditions. The given values are the mean number of RLU and SEMs from five independent experiments. Statistical analysis was performed using two-way ANOVA and the Bonferroni posttest. ***, $P < 0.001$ compared to the mean values obtained in DMEM-HEPES.

(vol/vol) nonessential amino acids (Invitrogen) at 37°C in a 5% CO_2 atmosphere. HeLa cells were dispensed in a 96-well cell culture plate (5×10^3 cells/well) for 24 h and then infected at a multiplicity of infection (MOI; number of bacteria per HeLa cell at the onset of infection) of 100 with the *E. coli* strains, which had previously been pregrown in the appropriate medium. At 4 h postinoculation, the cells were washed 3 times with Hanks' balanced salt solution (HBSS; Gibco) and incubated in cell culture medium for 72 h with 200 mg/ml gentamicin before fixation (4% formaldehyde) and protein staining with methylene blue (1% [wt/vol] in 0.01 M Tris-HCl). The methylene blue was extracted with 0.1 N HCl. Staining was quantified by measurement of the OD_{660}.

Electrophoretic mobility shift assay (EMSA). PCR products were generated by using genomic DNA from *E. coli* strain M1/5 as the template and the primers JPN42/JPN41 (*clbA*, 343 bp), clbC-up/clbC-rev (*clbC*, 219

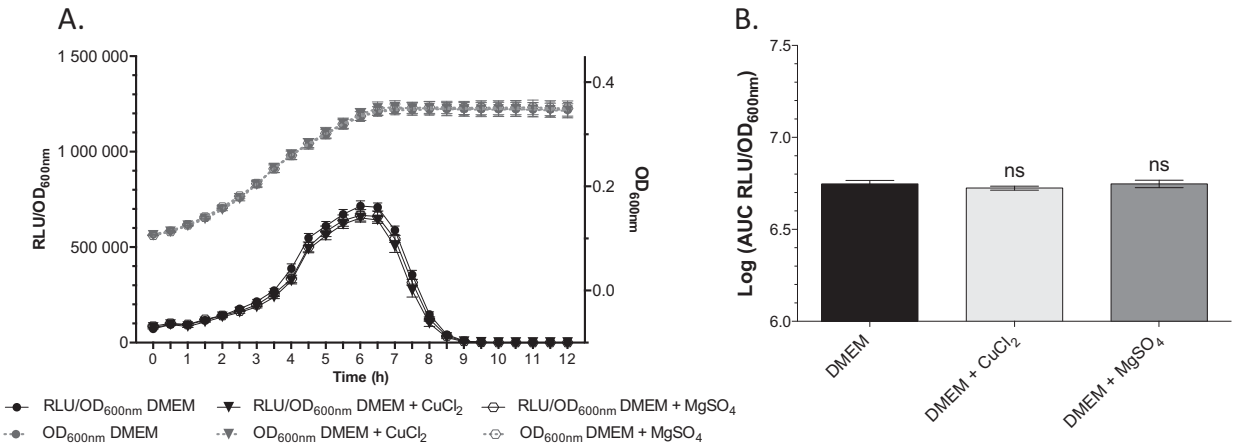

FIG 2 The transcription of *clbA* is not modulated by Cu^{2+} or Mg^{2+}. Growth curves (OD_{600}) and the RLU/OD_{600} of *E. coli* Nissle 1917 *clbA-lux* grown at 37°C in DMEM-HEPES and DMEM-HEPES supplemented with either 100 μM $MgSO_4$ or 100 μM $CuCl_2$ (A) and the associated AUC (B). The given values are the mean number of RLU and SEMs from at least three independent experiments. Statistical analysis was performed by comparison of the values to the mean values obtained in DMEM-HEPES using two-way ANOVA and the Bonferroni posttest. ns, not significant.

bp), and fyuA-up/fyuA-rev (*fuyA*, 648 bp) (Table 1). The PCR products were gel purified using a Wizard SV gel and PCR cleanup system (Promega). A 20-μl binding reaction mixture containing recombinant Fur protein (MyBioSource, USA), 50 μg ml^{-1} of poly(dI-dC), 4 μl of 5× binding buffer (50 mM Tris-HCl, pH 7.5, 200 mM KCl, 5 mM $MgCl_2$, 0.625 mM $MnCl_2$, 25% [vol/vol] glycerol, 2.5 mM dithiothreitol, 500 μg ml^{-1} bovine serum albumin), and the DNA probe (10 nM) was incubated at room temperature for 30 min, loaded onto a 4% Tris-borate polyacrylamide gel,

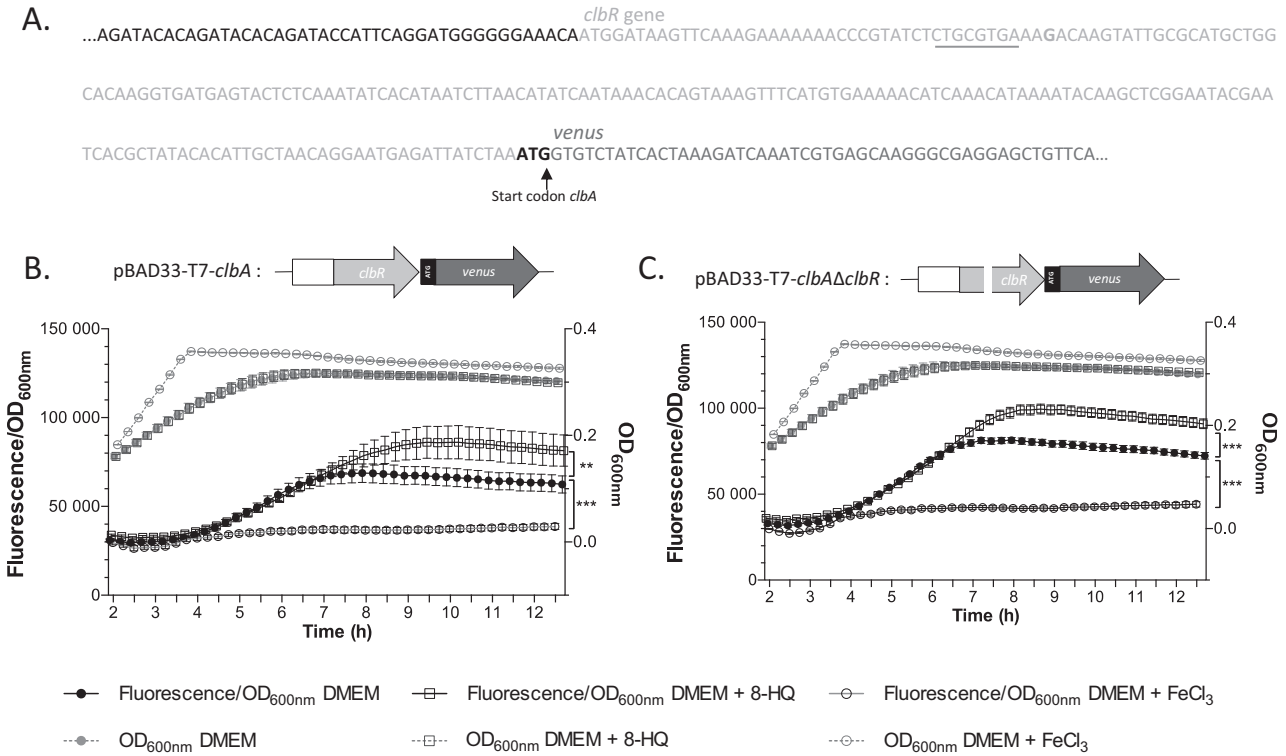

FIG 3 The transcription of *clbA* is dependent on the iron concentration in a ClbR-independent manner. (A) Sequence of the construction of the translational *Venus* fusion of *clbA* (*clbA-Venus* fusion). Two different constructs, one containing the wild-type *clbR* gene and one containing an inactive *clbR* gene, were generated. The underlined sequence was mutated to inactivate *clbR*. The wild-type and the mutated constructs were cloned into plasmid pBAD33-T7, and the plasmid was transformed into strain Nissle 1917 in which *clbA* was mutated. (B and C) Schematic representation of the plasmid construct obtained and results of the quantification of fluorescence of strain Nissle 1917 in which *clbA* was mutated and transformed with the plasmid containing the wild-type construct (B) or the mutated one (C). The fluorescence was measured in DMEM-HEPES or DMEM-HEPES supplemented with 100 μM $FeCl_3$ or 40 μM 8-HQ. The given values are the mean fluorescence/OD_{600} and SEMs three independent experiments. Statistical analysis was performed by comparison of the values to the mean values obtained in DMEM-HEPES using two-way ANOVA and the Bonferroni posttest. ***, $P < 0.001$; **, $P < 0.01$.

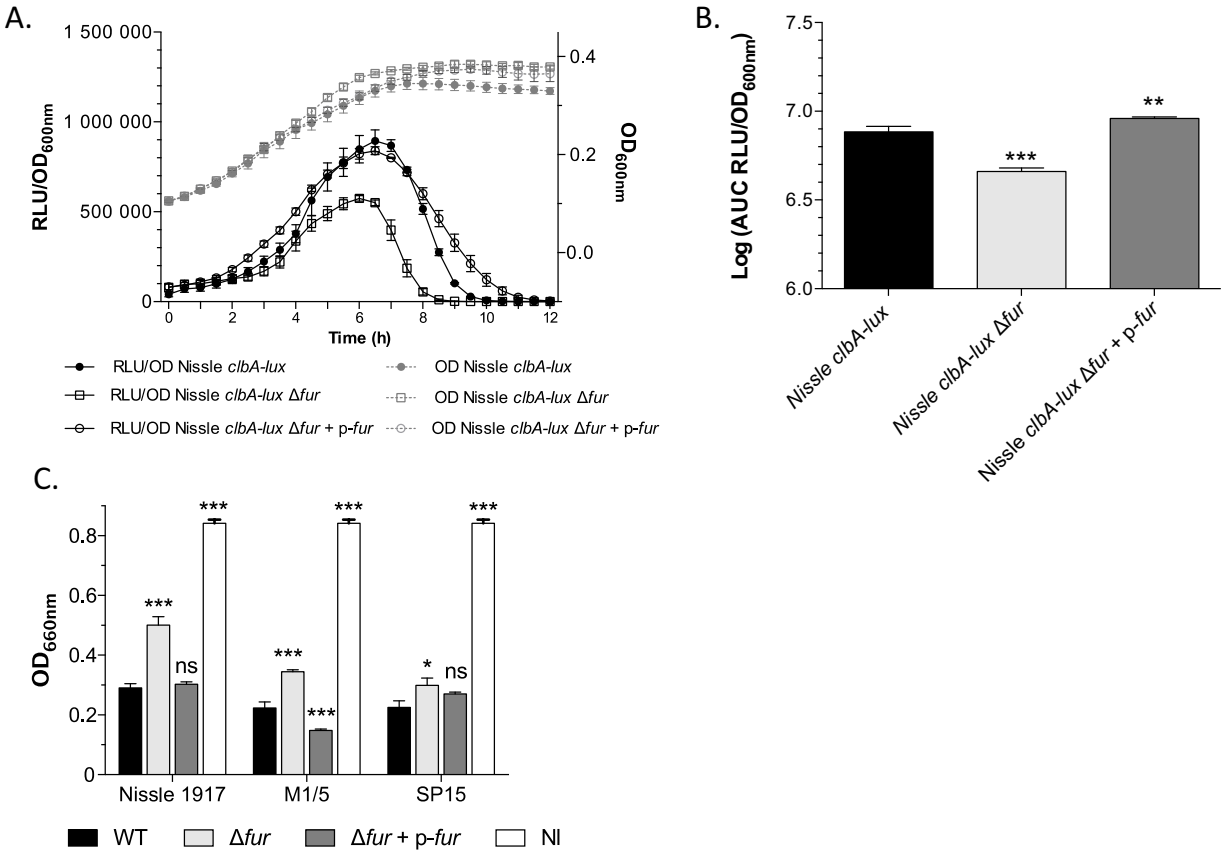

FIG 4 Fur regulates the transcription of *clbA* and colibactin biosynthesis. (A) Growth curves (OD_{600}) and the RLU/OD_{600} of strains Nissle 1917 *clbA-lux*, Nissle 1917 *clbA-lux* Δ*fur*, and Nissle 1917 *clbA-lux* Δ*fur*+p-*fur* grown at 37°C in DMEM-HEPES. The given values of the number of RLU and SEMs resulted from three independent experiments. (B) AUC of the RLU/OD_{600} of *clbA* in the three different strains. (C) Quantification of colibactin production. The production of colibactin by the *E. coli* strains and derivatives was determined by quantification of megalocytosis as previously described (15). The multiplicity of infection was 100 bacteria per cell. Three distinct genetic contexts of *E. coli* were investigated: Nissle 1917 (a probiotic strain), M1/5 (a commensal strain), and SP15 (a strain isolated from a patient with neonatal meningitis). Both cells that were not infected (NI) and cells that were infected with *E. coli* strains that do not produce colibactin gave the same quantitative results (data not shown). The given quantification values are represented as the mean values and SEMs from three independent experiments. Statistical analysis was performed by comparison of the values to the mean values obtained for wild-type (WT) strains using two-way ANOVA and the Bonferroni posttest. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

and electrophoresed in 0.5× Tris-borate, pH 7.5, containing 0.2 mM $MnCl_2$ at 100 V. The gels were stained with ethidium bromide.

Statistical analysis. Statistical analyses were conducted using GraphPad Prism (version 6.0) software. The mean with the standard error of the mean (SEM) is shown in the figures, and *P* values were calculated using a one-way or two-way analysis of variance (ANOVA) followed by a Bonferroni posttest, unless otherwise stated. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Iron availability modulates *clbA* transcription. In order to test whether the promoter activities of the genes located in the *pks* island (see Fig. S1A in the supplemental material) were dependent on iron availability, *E. coli* strain Nissle 1917 harboring transcriptional luciferase fusions with the genes *clbA*, *clbB*, *clbQ*, and *clbR*, four genes involved in colibactin biosynthesis, were studied (Table 1; see also Fig. S1A and B) (22). These strains were grown in chemically defined DMEM-HEPES, DMEM-HEPES supplemented with 100 μM $FeCl_3$, and DMEM-HEPES supplemented with an iron chelator, 8-hydroxyquinoline (8-HQ; 40 μM). The ranges of concentrations of the chelator and the iron supplement were analyzed, and the highest concentrations of substances which did not alter bacterial growth were chosen. The transcription rates of the genes *clbA*, *clbB*, *clbQ*, and *clbR* were determined as the number of OD_{600}-standardized relative luminescence units (RLU) (Fig. 1).

This analysis revealed that only *clbA* was significantly transcribed in DMEM-HEPES, as previously reported (22) (compare Fig. 1A to Fig. 1B to D). During the bacterial growth kinetics, a continuous increase in bioluminescence emission was observed to reach a maximal value at early stationary phase of growth, followed by a decrease of transcription (Fig. 1A).

The transcription of the genes *clbB*, *clbQ*, and *clbR* was not significantly modified when the culture medium was iron depleted (DMEM-HEPES supplemented with 40 μM 8-HQ) or iron replete (DMEM-HEPES supplemented with 100 μM $FeCl_3$) (Fig. 1B, C, and D, respectively). In contrast, under iron-depleted conditions, the expression of *clbA* was enhanced and maintained longer than that in DMEM-HEPES (Fig. 1A). Under iron-supplemented conditions, *clbA* transcription was strongly repressed (Fig. 1A). Calculation of the area under the curve (AUC) of the relative number of OD_{600}-standardized relative luminescence units (RLU/

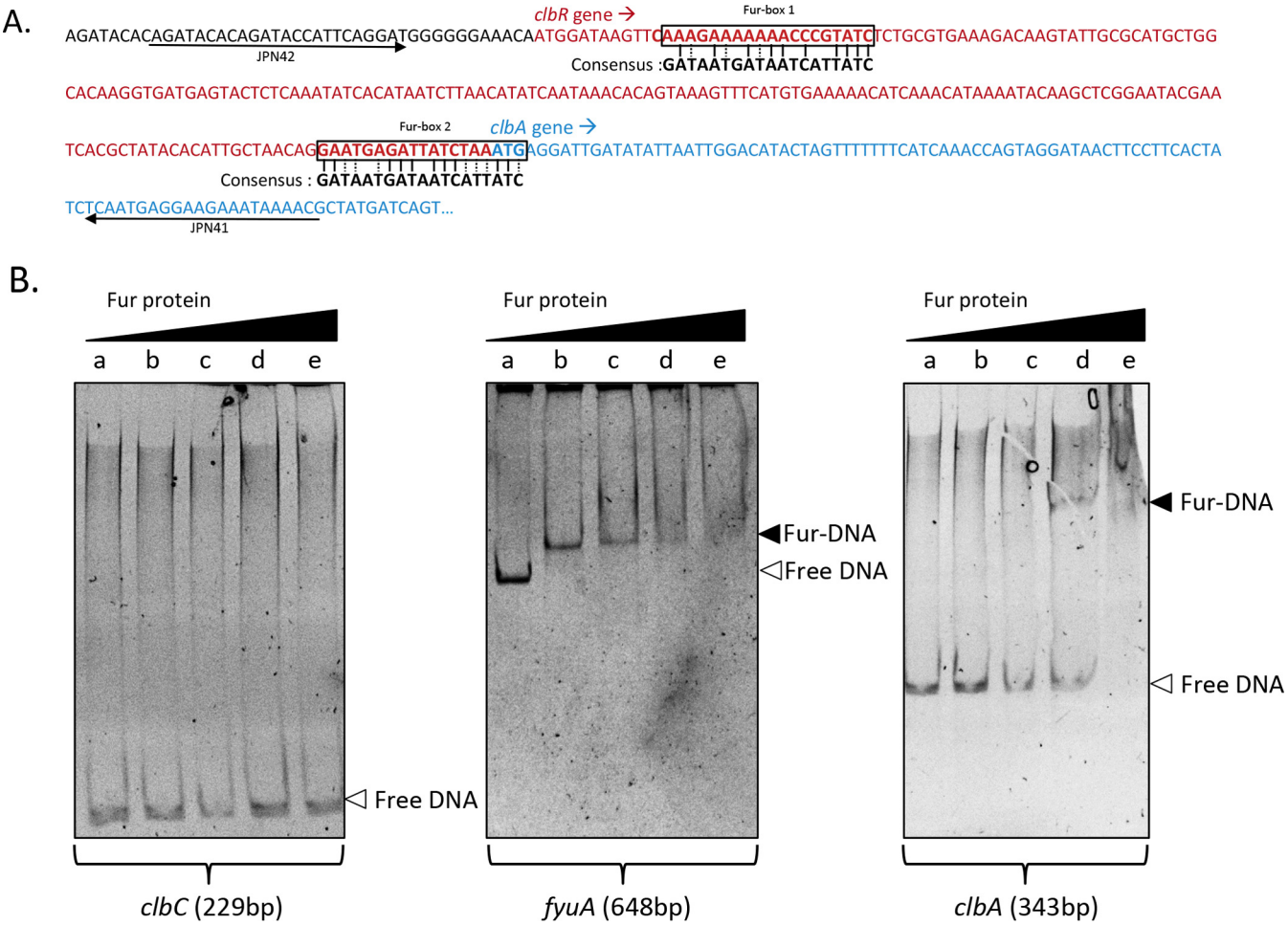

FIG 5 Fur binds to the *clbA* promoter. (A) Identification and localization of the two putative Fur boxes located upstream of *clbA*. (B) EMSA was performed in the presence of 0 μM (lanes a), 0.25 μM (lanes b), 0.5 μM (lanes c), 1 μM (lanes d), and 4 μM (lanes e) Fur protein and 10 ng of the PCR product amplified with primers JPN41/JPN42. Three PCR products were tested: a negative control (*clbC*, 229 bp), a positive control (*fyuA*, 648 bp), and the promoter region upstream of *clbA* (343 bp).

OD_{600}) confirmed the iron-dependent differential *clbA* expression (Fig. 1E). Supplementation with an alternative iron chelator (desferrioxamine [DFO], 0.2 μM) or an alternative iron source ($FeSO_4$, 100 μM) resulted in similar patterns (see Fig. S2 in the supplemental material).

In order to test whether other divalent metals could influence *clbA* transcription, we monitored the expression of *clbA* in strain Nissle 1917 *clbA-lux* grown in DMEM-HEPES supplemented with copper ($CuCl_2$, 100 μM) or magnesium ($MgSO_4$, 100 μM) (Fig. 2). This revealed that the transcription of *clbA* was not altered in the presence of Mg^{2+} or Cu^{2+}.

Altogether, these results show that *clbA* transcription is regulated by iron availability in the medium.

The iron-dependent transcription of *clbA* is independent of the LuxR-like protein ClbR. The gene *clbR* encodes a LuxR-like protein that exhibits a helix-turn-helix DNA-binding motif, which is suspected to be a regulator of colibactin biosynthesis (22). Moreover, cotranscription was previously demonstrated for the genes *clbR* and *clbA* in DMEM-HEPES medium with 5% fetal bovine serum (22). Our results described above showed that only *clbA* transcription and not *clbR* transcription is regulated by iron. To test whether ClbR is involved in the iron-dependent regulation of *clbA*, the *clbA* gene was transcriptionally fused with an alternative reporter gene, i.e., the *Venus* gene (coding for a variant of yellow fluorescent protein of *Aequorea victoria*) in plasmid pBAD33-T7-*Venus* to produce pBAD33-T7-*clbA*. The *clbR* gene was inactivated to produce plasmid pBAD33-T7-*clbA*Δ*clbR*. The resulting plasmids, pBAD33-T7-*clbA* and pBAD33-T7-*clbA*Δ*clbR*, were transformed into strain Nissle 1917 Δ*clbA*. Monitoring of the fluorescence of the Venus protein transcriptionally fused with the *clbA* gene in strain Nissle 1917 Δ*clbA*+pBAD33-T7-*clbA* confirmed the iron-dependent regulation of *clbA* (Fig. 3A and B). Monitoring of the fluorescence of strain Nissle 1917 Δ*clbA*+pBAD33-T7-*clbA*Δ*clbR* under iron-supplemented and iron-chelated conditions revealed that *clbA* transcription was regulated by the iron concentration in the absence of functional ClbR (Fig. 3A and C). These results show that ClbR is not involved in the iron-dependent regulation of *clbA*.

Fur positively regulates the production of colibactin. Because the global response to iron availability in bacteria relies on the ferric uptake regulator (Fur) protein, we tested whether the iron-dependent regulation of *clbA* transcription was mediated by Fur. We mutated the *fur* gene in strain Nissle 1917 *clbA-lux*. The com-

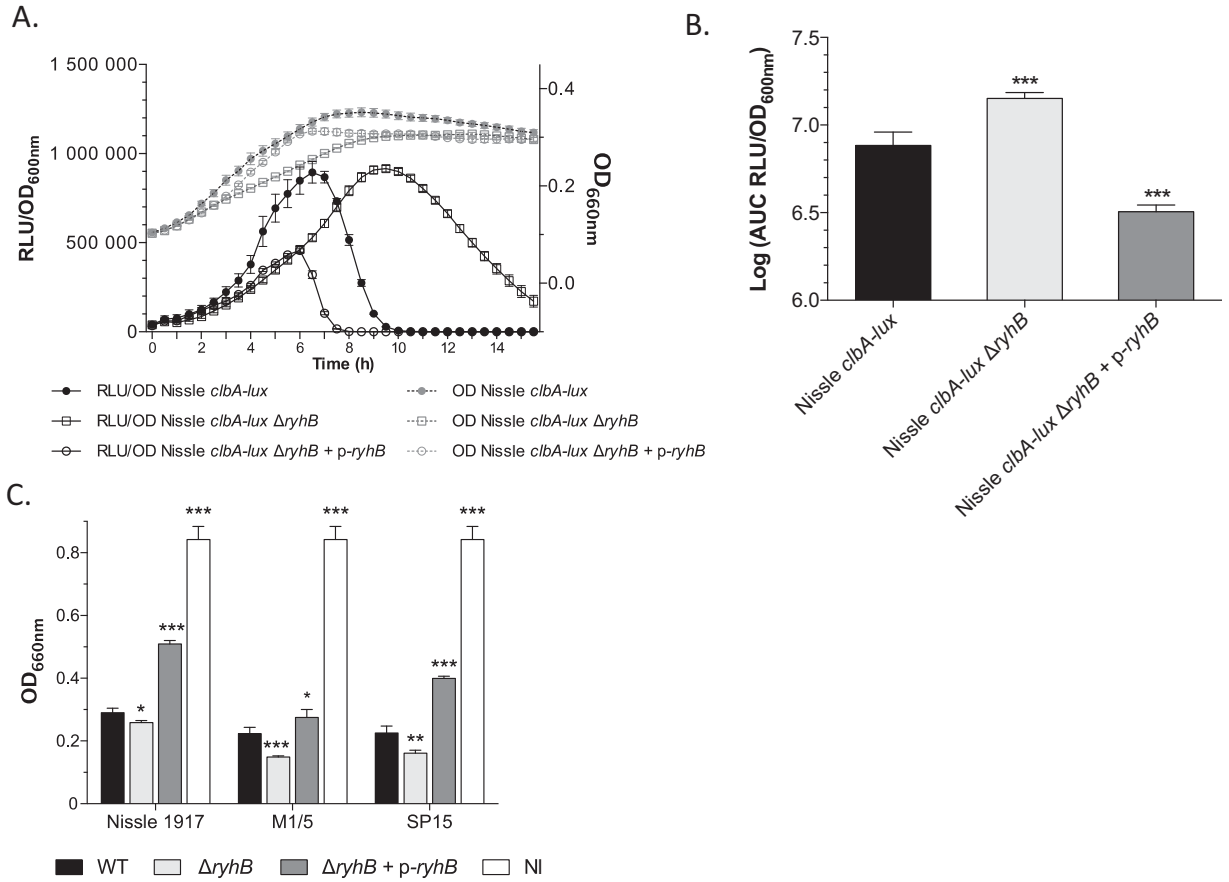

FIG 6 RyhB regulates the transcription of *clbA* and colibactin biosynthesis. (A) Growth curves (OD_{600}) and the RLU/OD_{600} of strains Nissle 1917 *clbA-lux*, Nissle 1917 *clbA-lux* Δ*ryhB*, and Nissle 1917 *clbA-lux* Δ*ryhB*+p-*ryhB* grown at 37°C in DMEM-HEPES. The given values are the mean number of RLU and SEMs from three independent experiments. (B) AUC of the RLU/OD_{600} of *clbA* in the three different strains. (C) Quantification of colibactin production. The production of colibactin by *E. coli* strains and its derivatives was determined by quantification of megalocytosis as previously described (14). The multiplicity of infection was 100 bacteria per cell. Three distinct genetic contexts of *E. coli* were investigated: Nissle 1917 (a probiotic strain), M1/5 (a commensal strain), and SP15 (a strain isolated from a patient with neonatal meningitis). Both cells that were not infected (NI) and cells that were infected with *E. coli* strains that do not produce colibactin gave the same quantitative results (data not shown). The given quantification values are represented as mean values and SEMs from three independent experiments. Statistical analysis was performed by comparison of the values to the mean values obtained for wild-type strains using two-way ANOVA and the Bonferroni posttest. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

plemented strain Nissle 1917 *clbA-lux* Δ*fur*+p-*fur*, where the plasmid-borne wild-type *fur* gene was transformed into strain Nissle 1917 *clbA-lux* Δ*fur*, was also constructed (Table 1). Monitoring of the expression of *clbA* in the resulting strains (Fig. 4A and B) revealed that the transcription of *clbA* was significantly decreased in the strain in which *fur* was mutated (Fig. 4A and B). Transformation of the strain in which *fur* was mutated with a plasmid carrying the functional wild-type *fur* gene (Fig. 4A and B) totally restored the expression of *clbA*. This indicates that the transcription of *clbA* is positively regulated by Fur.

Since ClbA is required for the production of colibactin, the role of Fur in the synthesis of colibactin was then investigated. Eukaryotic cells were infected with *pks*$^+$ *E. coli* strains in which *fur* was mutated or not. Three different genetic contexts were analyzed: strains Nissle 1917 (a probiotic strain), M1/5 (a commensal strain), and SP15 (a pathogenic strain isolated from a patient with neonatal meningitis) (Table 1). The genotoxic effect of colibactin was monitored by quantification of megalocytosis, as previously described (14). This revealed that all the Δ*fur* mutants displayed a genotoxicity significantly reduced compared to that of the wild-type strains (Fig. 4C). Transformation of the strains in which *fur* was mutated with a plasmid carrying the functional wild-type *fur* gene resulted in restoration of the megalocytosis phenotype (Fig. 4C). Altogether, these results evidence that Fur positively regulates colibactin production.

To determine whether Fur could regulate *clbA* directly and/or indirectly, we investigated a putative direct binding of Fur to *clbA*. We detected two putative Fur-binding sites in the DNA sequence of the *clbA* promoter (Fig. 5A). We tested whether purified Fur protein bound to its putative binding sites in the *clbA* promoter region (Fig. 5B). EMSA showed that migration of the 343-bp DNA fragment was retarded when it was incubated with the Fur protein. This suggests that Fur can regulate *clbA* expression through direct binding.

RyhB negatively regulates the production of colibactin. Because the second major regulator of bacterial iron metabolism is the small regulatory noncoding RNA RyhB, we investigated whether RyhB was involved in the iron-mediated regulation of *clbA* transcription. The gene *ryhB* was inactivated in strain Nissle 1917 *clbA-lux*, and the complemented strain was also engineered

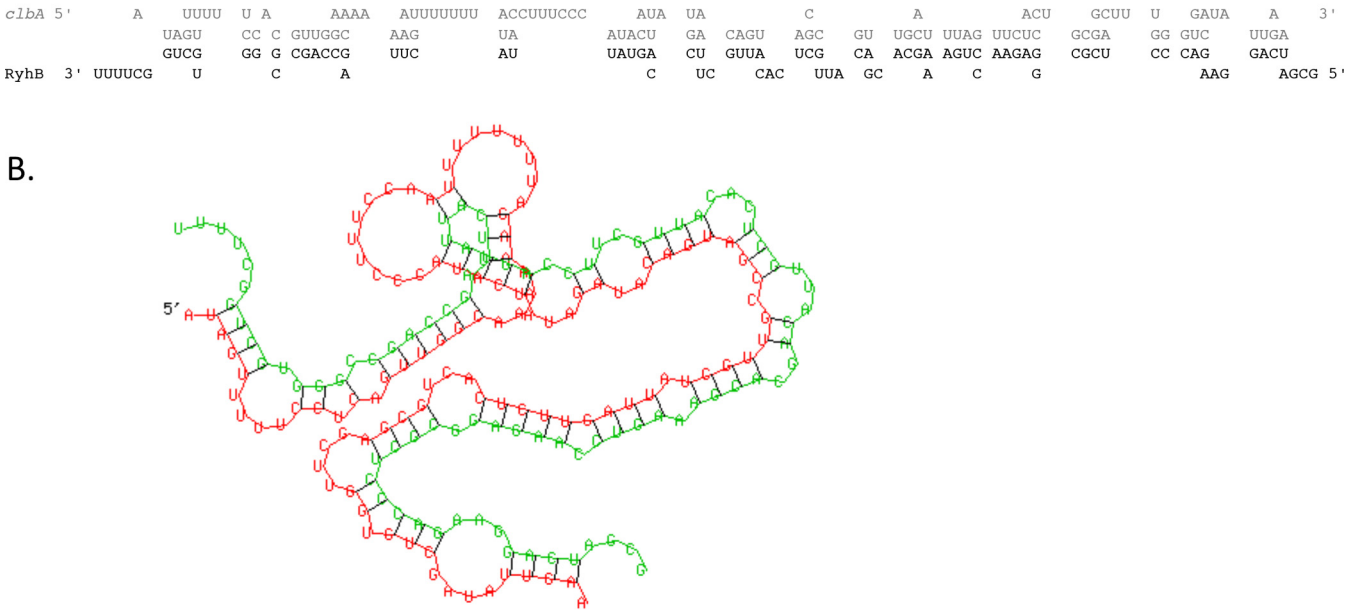

FIG 7 RyhB seems to interact directly with the *clbA* gene. The RNA Hybrid computational program predicted a region of potential interaction between RyhB and *clbA*. The nucleotide sequence involved in the interaction between *clbA* (gray) and RyhB (black) (A) and the two-dimensional structure of RyhB (green) bound to *clbA* (red) (B) are shown. The predicted pairing region with RyhB began at nucleotide 238 in the *clbA* open reading frame (minimal free energy, −51.3 kcal/mol).

(Table 1). Monitoring of the bioluminescence in the resulting strain, Nissle 1917 *clbA-lux* Δ*ryhB*, revealed that the level of transcription of *clbA* was enhanced and maintained longer than it was in strain Nissle 1917 *clbA-lux* (Fig. 6A and B). Overexpression of the wild-type *ryhB* gene expressed from a high-copy-number plasmid in strain Nissle 1917 *clbA-lux* Δ*ryhB* resulted in the repression of *clbA* transcription (Nissle 1917 *clbA-lux* Δ*ryhB*+p-*ryhB*) (Fig. 6A and B). These data evidence that RyhB plays a critical role in the transcription of *clbA*.

The role of RyhB in the synthesis of colibactin was then investigated. Eukaryotic cells were infected with *pks*$^+$ *E. coli* strains in which *ryhB* was mutated or not. The genotoxic effect of colibactin, quantified by megalocytosis, revealed that all the Δ*ryhB* mutants displayed significantly increased genotoxicity compared to the wild-type strains (Fig. 6C). Overexpression of *ryhB* in strains in which the *ryhB* gene was mutated resulted in a decrease in the level of the colibactin-associated genotoxic effect (Fig. 6C). Altogether, these results evidence that RyhB negatively regulates colibactin production under these conditions.

To determine whether RyhB could regulate *clbA* directly and/or indirectly, we investigated a putative direct pairing of RyhB to the *clbA* promoter. The RNA Hybrid computational program (23) predicted a region of potential pairing between RyhB and *clbA* (Fig. 7). The region of pairing with *clbA* was located 238 to 344 nucleotides downstream from the translational start (minimal free energy, −51.3 kcal/mol) (Fig. 7) in the reading frame of *clbA*. This suggests that *ryhB* could regulate *clbA* through direct binding.

DISCUSSION

This work highlights that the transcription of *clbA*, the gene encoding the PPTase involved in colibactin production, is regulated by iron availability (see our model in Fig. 8). Therefore, this work provides new insights into the transcriptional regulation of *clbA*, as only one study on its regulation has been published previously (22). In addition to carbon source and growth phase (22), our work demonstrates that *clbA* is regulated by iron availability. Moreover, this regulation appears to be independent of ClbR, the LuxR-like protein predicted to regulate colibactin biosynthesis (22), although the *clbR* gene was demonstrated to be coexpressed with *clbA* under particular culture conditions (22). This suggests that *clbA* could be expressed from a second promoter specifically activated under specific conditions of iron availability. This iron-dependent regulation of *clbA* transcription could constitute a fitness advantage for the bacteria, as we previously demonstrated that ClbA can also sustain siderophore biosynthesis (20).

This work highlights that Fur is positively involved in the production of the virulence factor colibactin through the regulation of *clbA*. Fur is a global transcriptional regulator that controls the transcription of over 90 genes involved in iron uptake, storage, and metabolism. Only a few studies have reported the involvement of Fur in the regulation of the biosynthesis of toxins in *E. coli*. The *stx* genes, encoding Shiga toxin in enterohemorrhagic *E. coli* (24, 25), and *hly* plasmids, encoding hemolysin (26), are directly regulated by Fur. In addition to its well-known role as a repressor, Fur was characterized as a positive regulator of gene expression, such as the expression of *acnA*, *fumA*, *ftnA*, *bfr*, and *sodB* (27).

Our work also evidenced that RyhB regulates *clbA* transcription, as we observed that *clbA* transcription was upregulated when the *ryhB* gene was inactivated, demonstrating that *clbA* is downregulated by RyhB. RyhB is a small RNA that recruits RNase E and facilitates the degradation of mRNA targets (27, 28). Whereas only a few studies have demonstrated the impact of RyhB on virulence

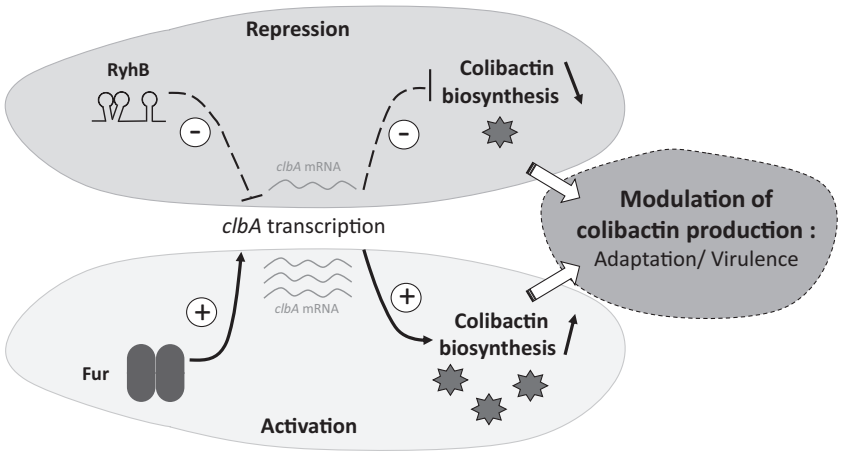

FIG 8 Model for the regulation of the transcription of *clbA* by Fur and RyhB. The biosynthesis of the genotoxin colibactin requires the enzymatic activity of the PPTase ClbA. The transcription of *clbA* is dependent on both the ferric uptake regulator (Fur) and the small regulatory noncoding RNA RyhB. This makes iron a key environmental factor contributing to the regulation of colibactin production.

in pathogenic *E. coli*, for instance, through siderophore production (29), several studies have demonstrated that this sRNA is implicated in the virulence-associated processes of other pathogenic bacteria, such as *Shigella flexneri* (2) or *Shigella dysenteriae* (30). This study provides new evidence that RyhB is involved in the production of a virulence factor in pathogenic *E. coli* through the regulation of *clbA* transcription, which leads to the modulation of colibactin production.

Here we demonstrated that modulation of the expression of *clbA* via regulators of iron homeostasis leads to the modulation of colibactin production. The mutation of *ryhB* in a colibactin-producing *E. coli* strain leads to an increase in the level of colibactin production, whereas the mutation of *fur* results in a decrease in the level of colibactin production in three different genetic contexts of *E. coli*, in which the strains belonged to the B2 phylogenetic group. We confirmed that this regulation occurred in three different strains; however, the quantitative differences observed could be explained by the genetic diversity between strains. Colibactin was demonstrated to be a virulence factor, for example, in neonatal systemic infections (19), but it is also associated with the development of colorectal cancer (31–33). The fact that colibactin is regulated by the two major regulators of iron homeostasis could link iron availability in the gut and blood and *E. coli*-mediated carcinogenesis and systemic infections, respectively. Moreover, the main reservoir of *E. coli* is the intestine and, more precisely, the colon. A recent publication confirmed the emergence of the B2 group of *E. coli* in developed countries (34). 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* isolate is located in an appropriate site in the gut. This iron-dependent regulation could be one reason for the emergence of the B2 group *E. coli* strains, as we know that there is cross talk between colibactin and siderophores. Integration of 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 the accurate production of colibactin and siderophores, so that the bacteria can adapt to the competitive environment in the gut.

Further understanding of how *E. coli* senses environments to coordinate the expression of *clbA* and the mechanism of regulation of the biosynthesis of these virulence factors may uncover novel pathways for the development of potential targets against this pathogenic enterobacterium.

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We have no conflict of interest to declare.

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