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Expression of Hemin Receptor Molecule ChuA Is Influenced by RfaH in Uropathogenic *Escherichia coli* Strain 536

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The outer membrane protein ChuA responsible for hemin utilization has been recently identified in several pathogenic *Escherichia coli* strains. We report that the regulatory protein RfaH influences ChuA expression in the uropathogenic *E. coli* strain 536. In an *rfaH* mutant, the *chuA* transcript as well as the ChuA protein levels were significantly decreased in comparison with those in the wild-type strain. Within the *chuA* gene, a consensus motif known as the JUMPStart (just upstream of many polysaccharide associated gene starts) sequence was found, which is shared by RfaH-affected operons. Furthermore, the presence of two different subclasses of the *chuA* determinant and their distribution in *E. coli* pathogroups are described.

The availability of iron, an essential nutrient for bacterial growth, is severely limited in mammalian hosts. In order to compete with the host for iron, pathogenic bacteria have developed different mechanisms to acquire this essential growth factor (10). Low-molecular-weight chelators (siderophores) are secreted by several pathogens. These molecules liberate Fe^{3+} from host carriers and transport it into bacterial cells. Alternatively, many pathogenic bacteria can directly utilize iron-containing host compounds through specific receptors. Several gram-negative pathogens, e.g., *Haemophilus influenzae* type b (6), yersiniae (34, 37), *Vibrio cholerae* (26), neisseriae (17, 35), and *Shigella dysenteriae* (21), express outer membrane proteins involved in the utilization of heme and its protein complexes as iron sources. In *Escherichia coli* O157:H7 the gene *chuA*, which codes for a 69-kDa outer membrane protein responsible for heme uptake, was recently identified (38). The *chuA* nucleotide sequence shows high homology to that of the formerly described *shuA* gene of *S. dysenteriae* type 1 (40). The gene is part of a larger locus, termed the heme transport locus, which appears to be widely distributed among pathogenic *E. coli* strains (41). This locus contains eight open reading frames and is located at 78.7 min of the *E. coli* K-12 chromosome.

The ability to use heme and/or hemoglobin might be especially advantageous to pathogenic bacteria. These pathogens often secrete cytotoxins, which gain access to the intracellular heme reservoir besides initiating tissue invasion. Cytotoxin production coupled with the capability to utilize heme and/or hemoglobin could serve as an effective iron acquisition strategy during the progression of infection.

RfaH regulates the transcription of long operons probably at the level of transcription antitermination, hence suppressing operon polarity (2, 18). These operons share a conserved motif, which was identified for the first time in polysaccharide-associated operons and was therefore termed the JUMPStart (for just upstream of many polysaccharide-associated gene starts) sequence (12). The most-conserved part of this 39-bp motif is an 8-bp sequence termed the *ops* element (for operon polarity suppressor), which is always associated with a direct repeat that shows less similarity to the standard element (2). Deletion of the *ops* element and/or other parts of the JUMPStart sequence results in transcriptional polarity of the affected operons (19, 24). A similar transcriptional pattern is observed in *rfaH* mutants, suggesting that the regulation of these operons by RfaH is dependent on the presence of the JUMPStart motif. In this study we investigated the effect of RfaH on the expression of the *E. coli* hemin receptor protein ChuA.

Bacterial strains and culture conditions. The uropathogenic *E. coli* strain 536 was isolated from a patient with acute pyelonephritis (3). In the mutant strain 536*rfaH*::*cat*, the *rfaH* gene was inactivated by insertion of a chloramphenicol acetyltransferase (*cat*) cassette. The insertion was performed by allelic exchange as previously described (23). *trans*-complementation of *rfaH* was achieved by supplying the mutant strain with the plasmid pSMK1, which carried *rfaH* together with its promoter region cloned into the vector pGEM-T Easy (Promega). The strains used in Southern hybridization experiments are listed in Table 1. The enterohemorrhagic *E. coli* (EHEC) strain 95004730 and the enteroaggregative *E. coli* (EAggEC) strain DPA065 were provided by Robert Pringle (Victorian Infectious Diseases Reference Laboratory, North Melbourne, Australia) and Anna Giammanco (Dipartimento di Igiene e Microbiologia, University of Palermo, Palermo, Italy), respectively. The origins of all other *E. coli* wild-type strains are referenced in Table 1. Bacteria were grown routinely in Luria-Bertani broth or Luria-Bertani broth solidified with 1.5% agar (Difco, Detroit, Mich.). In iron-restricted studies, a 0.4 mM concentration of the iron chelator 2,2′-dipyridyl (Sigma, Deisenhofen, Germany) was added to the media. When appropriate, the medium was supplemented with the following antibiotics at the indicated concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml.

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TABLE 1. Occurrence of *chuA* and the two distinct *chuA* upstream regions among pathogenic *E. coli* strains

Strain	Serotype	Patho-group[a]	Hybridization with[b]:			Reference or source
			Probe-1 (*chuA*)[c]	Probe-2	Probe-3	
HB101		K-12	−	−	−	5
536	O6:K15:H31	UPEC	12	+	−	3
J96	O4:K6	UPEC	12	+	−	13
AD110	O6:K2	UPEC	12	+	−	39
764	O18:K5:H5/11	UPEC	12	+	−	27
2980	O18:K5:H5/11	UPEC	12	+	−	27
RZ439	O6:K5	UPEC	12	+	−	43
RZ441	O6:K5	UPEC	12	+	−	43
RS218	O18:K1:H7	NBM	12	+	−	33
IHE3034	O18:K1:H7/9	NBM	12	+	−	15
IHE3036	O18:K1:H7/9	NBM	12	+	−	15
EDL933	O157:H7	EHEC	11	−	+	25
9167/91	O157:H7	EHEC	11	−	+	7
5159/91	O157:H7	EHEC	11	−	+	31
86-24	O157:H7	EHEC	11	−	+	8
E32511	O157:H7	EHEC	11	−	+	11
6578/93	O157:H7	EHEC	11	−	+	31
SF493/89	O157:H$^-$	EHEC	11	−	+	14
3574/92	O157:H$^-$	EHEC	11	−	+	7
3978/91	O157:H$^-$	EHEC	11	−	+	30
5291/92	O157:H$^-$	EHEC	11	−	+	30
2907/97	O55:H6	EHEC	11	−	+	This study
5720/96	O26:H$^-$	EHEC	−	−	−	42
3697/97	O26:H$^-$	EHEC	−	−	−	32
ED147	O26:H11	EHEC	−	−	−	29
5714/96	O103:H2	EHEC	−	−	−	32
ED142	O111:H$^-$	EHEC	−	−	−	29
78/92	O111:H$^-$	EHEC	−	−	−	36
95004730	O111:H$^-$	EHEC	−	−	−	RP[e]
E2348/69	O127:H6	EPEC	12	+	−	20
179/2	O55:H6	EPEC	12	+	−	This study
156A	O55:H7	EPEC	−	−	−	4
182A	O55:H7	EPEC	11	−	+	4
37-4	O55:H$^-$	EPEC	−	−	−	20
76-5	O143:HND	EIEC	11	−	+	This study
12860	O124:HND	EIEC	−	−	−	This study
EDL1284	ND[d]	EIEC	11	−	+	This study
C9221a	O6:K15:H16	ETEC	−	−	−	This study
DPA065	O119:HND	EAggEC	−	−	−	AG[f]
5477/94	O86:H7	EAggEC	−	−	−	This study
7484/94	O86:H18	EAggEC	−	−	−	This study
DDC4441	O128:HND	EAggEC	−	−	−	This study
17-2	O3:H2	EAggEC	−	−	−	40
5464/95	O3:H2	EAggEC	−	−	−	This study

[a] Abbreviations: UPEC, uropathogenic *E. coli*; NBM, newborn meningitis-causing *E. coli*; ETEC, enterotoxigenic *E. coli*.
[b] The probes used are described in Fig. 3.
[c] The numbers indicate the size of the fragments hybridized with the probe (in kilobases) after digestion of chromosomal DNA with *Bgl*I.
[d] ND, not determined.
[e] RP, Robert Pringle (Victoria Infectious Diseases Reference Laboratory).
[f] AG, Anna Giammanco (Dipartimento di Igiene e Microbiologia, University of Palermo).

Expression of ChuA is decreased in the *rfaH* mutant of strain 536. The ChuA protein levels expressed in *E. coli* 536 and its derivatives were determined by Western blotting (Fig. 1A). Whole-cell extracts obtained from bacteria grown in normal and iron-restricted media were separated on a 10% polyacrylamide gel and were blotted onto a nitrocellulose membrane. The blocked membranes were treated with an anti-HemR antiserum (kindly provided by J. Heesemann) and were developed as described elsewhere (28). HemR is the hemin receptor protein of *Yersinia enterocolitica*. The HemR antiserum was proven to be cross-reactive with ChuA of *E. coli* 536 (data not shown). The quantity of ChuA protein was strongly reduced in the *rfaH*-negative strain compared to the wild type. *trans*-complementation of the mutant strain with *rfaH* (on pSMK1) restored higher levels of ChuA. No ChuA protein was detectable in the absence of 2,2′-dipyridyl, indicating that expression of ChuA is dependent on the availability of free iron.

To investigate whether the altered expression of ChuA protein was a consequence of decreased *chuA* transcription in the *rfaH* mutant, we performed Northern blot analysis (Fig. 1B). Total RNA was isolated from bacteria harvested from iron-restricted medium using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Northern blot analysis was performed as described previously (1). Ten micrograms of isolated RNA per lane was separated on a 1.2% agarose-formaldehyde gel and was transferred overnight to a nylon membrane (Biodyne B; Pall Ltd., Portsmouth, England) by capillary blotting. The DNA probe specific for the 3′ end of *chuA* was generated by PCR using the primers 5′-GTCGCTTCTATACCAACTATTGGGTG-3′ and 5′-CCGTTACGACCATCCTGTG-3′ and was labeled with the ECL direct labeling system (Amersham-Pharmacia, Freiburg, Germany). Hybridization was carried out overnight at 42°C as described by Amersham-Pharmacia. Before luminography, the membrane was washed twice for 15 min in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.4% sodium dodecyl sulfate (SDS) (50°C) and then twice for 5 min in 2× SSC (20°C). The *chuA*-specific DNA probe hybridized to a 2.2 to 2.3-kb mRNA, which corresponds to the *chuA* transcript (Fig. 1B). The absence of an intact *rfaH* gene resulted in reduced levels of *chuA* mRNA; however, the length of the transcript was not altered. Overexpression of RfaH (536*rfaH*::*cat*

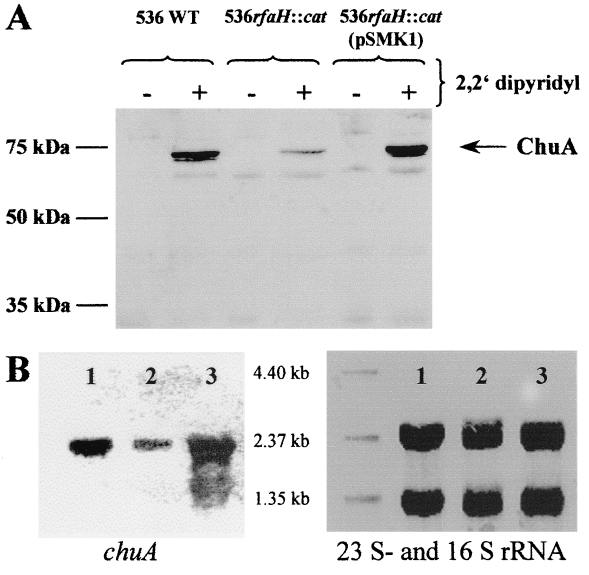

FIG. 1. Influence of RfaH on *chuA* expression of *E. coli* strain 536. (A) Detection of ChuA levels by Western blot analysis of whole-cell extracts of *E. coli* strain 536 and its derivatives using a HemR-specific antiserum. The strains were grown in the presence (+) or absence (−) of 0.4 mM 2,2′-dipyridyl. (B) Analysis of *chuA* transcript levels of *E. coli* strain 536 and its derivatives. An enhanced chemiluminescence-labeled *chuA*-specific probe was hybridized to total RNA isolated from strain 536 (lane 1), 536*rfaH*::*cat* (lane 2), 536*rfaH*::*cat* (pSMK1) (lane 3). The 23S and 16S rRNA were stained with 0.3% methylene blue after transfer of separated total RNA to a nylon membrane (internal control).

Operon	JUMPStart sequence (*ops*)
cps	CAGTGCGCTGGTAGCTGTTAAGCCAGG**GGCGGTAG**CGTC
kps	CAGTGTATTGGTAGCTGTTAAGCCAAG**GGCGGTAG**CGTG
rfb	CAGTGCTCTGGTAGCTGTTAAGCCAGG**GGCGGTAG**CGTG
tra	CGAGTGCCCTGTGCGTGAAAAGGGATG**GGCGGTAG**CGTG
rfa	CAGTATTCAGGTAGCTGTTGAGCCTGG**GGCGGTAG**CGTG
p152 *hly*	TCCCGGTTTACGGGTAGTTTCCGGAAG**GGCGGTAG**CATG
2001 *hly*	CCGGTTGATACGGGTAATTTCCGGAAG**GGCGGTAG**CATG
J96 *hlyI*/536 *hlyI*	GCTGGTTGATGACTGTTAATTCCAGAA**GGCGGTAG**TCTG
536 *hlyII*	CCGGTTGATACGGGTAATTTCCGGAAG**GGCGGTAG**CATG
536 *chu*	CTTGGCGGAACCCGCTATGACAGTTATC**GCGGTAG**CAGC
EDL933 *chu*	CTTGGCGGAACCCGCTATGACAGTTATC**GCGGTAG**CAGT

FIG. 2. Comparison of JUMPStart sequences from different *E. coli* operons. Boldface letters denote the *ops* element; underlined bases represent the imperfect repeats within the JUMPStart sequences. Accession numbers or references for the following sequences are as follows: *cps*, AF104912; *kps*, X53819; *rfb*, U09876; *tra*, U01159; *rfa*, M86935; p152 *hly*, M14107 and X07565; 2001 *hly*, reference 24; J96 *hly*, M10133; 536 *hlyI* and *hlyII*, G. Nagy and G. Blum-Oehler, unpublished data; 536 *chu*, AF280396; EDL933 *chu*, U67920.

carrying pSMK1) manifested in an increased *chuA* transcription compared to the level found in the wild-type strain. RfaH has been known as a regulator which influences the transcription of long operons encoding cell surface and extracellular components that are important for bacterial fertility and virulence. These include the *hly, rfa, rfb, tra, cps*, and *kps* operons that direct the synthesis of α-hemolysin, lipopolysaccharide core, O antigen, F factor, and group I and group II capsule, respectively (2). The hemin receptor ChuA is also anchored in the outer membrane of pathogenic *E. coli* strains and is considered to be a potential virulence factor. As the encoding gene (*chuA*) is transcribed as a monocistronic mRNA, the way RfaH is involved in transcriptional regulation of *chuA* transcription seems to be inconsistent with the present view that RfaH acts as a transcriptional antiterminator without affecting transcription initiation (18).

Coregulation of different determinants involved in pathogenicity is energetically advantageous for pathogenic bacteria. This is especially true for components of a complex system which are functionally related. α-Hemolysin expression and hemin uptake are both regulated by iron (16, 21), suggesting that the utilization of heme compounds liberated from eukaryotic cells is an important iron acquisition strategy during infection. Coupled regulation by RfaH gives further evidence that the function of the *E. coli* hemin uptake system (*chu*) is dependent on secreted hemolysin.

Sequence analysis of the *chuA* gene of *E. coli* strain 536. Sequencing of the *chuA* gene was performed from a cosmid clone of *E. coli* 536 using an ABI Prism 310 automatic sequencer. It was previously shown that RfaH-regulated operons carry a conserved region known as the JUMPStart sequence (2). Within the *chuA* gene of *E. coli* strain 536, a similar motif was identified. A comparison of this motif to JUMPStart sequences of other *E. coli* operons known to be regulated by RfaH is shown in Fig. 2. The 39-bp region found in the *chuA* gene is located 1,158 bp downstream of the start codon. It contains an *ops*-like motif with an additional conserved C base located downstream of the *ops* element. In the 5′ region of the JUMPStart sequence, a relatively well-conserved direct repeat could be identified with relevant spacing similar to those of other JUMPStart sequences.

The 1,983-bp coding region of *chuA* shows high homology to the corresponding sequences derived from *E. coli* O157:H7 (38) and *S. dysenteriae* (22). The potential promoter region is located about 300 bp upstream of the start codon, and is overlapped by a putative Fur box. The presence of this motif neighboring the promoter explains the observed effect of iron availability on ChuA protein levels. In contrast to the high homology between the coding regions of different *chuA* and *shuA* determinants, the *E. coli* 536-specific *chuA* upstream region showed less similarity to the corresponding regions of *E. coli* O157:H7 and *S. dysenteriae*. A 74-bp region located between the putative promoter and the start codon of the *E. coli* 536-specific determi-

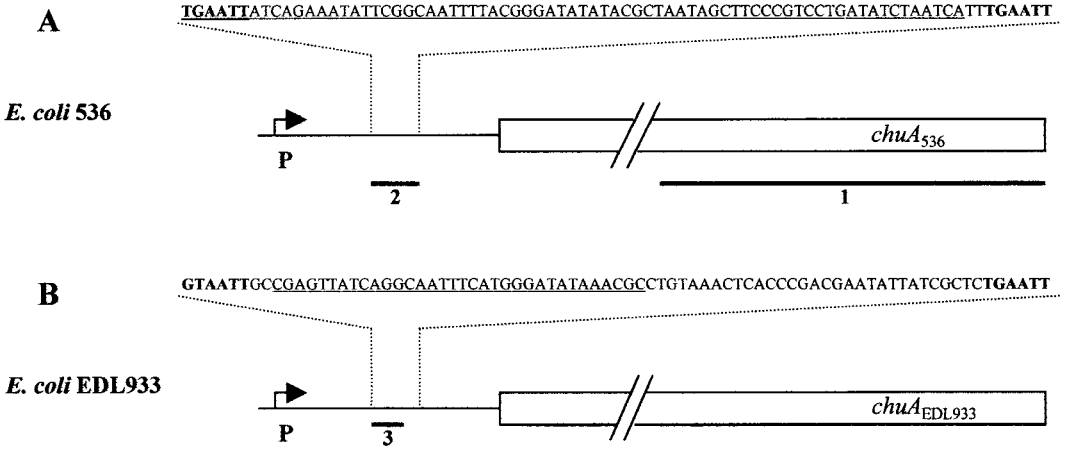

FIG. 3. Genetic map of the uropathogenic *E. coli* strain 536-specific *chuA* gene (A) and that of EHEC strain EDL933 (B). The *chuA* coding regions are indicated by boxes; the 5′ flanking regions are indicated by single lines. The arrows labeled with P denote the promoters. The sequences of the upstream element specific for strain 536 or strain EDL933 are given. Bases in boldface type represent the direct repeats flanking dissimilar regions. The numbers and thick lines denote the probes used for Southern hybridization (see text). The sequences of oligonucleotides used as probes are underlined.

nant is replaced by a totally different 73-bp motif in *S. dysenteriae* and *E. coli* O157:H7 (Fig. 3). In the uropathogenic strain, this region is flanked by 6-bp direct repeats that might have served as a site for recombination. In *E. coli* O157:H7, this region is bordered by similar, nevertheless imperfect, repeats.

To investigate the distribution of the two different identified 5′-flanking sequences of *chuA*, several *E. coli* strains representing different pathogroups were tested by Southern hybridization. Chromosomal DNA was isolated as described before (9). The DNA was digested with *Bgl*I prior to separation on a 0.8% agarose gel and subsequent transfer to a nylon membrane (Biodyne B; Pall Ltd.) The presence of *chuA* in the genomes was proven by hybridization of a 600-bp probe derived from the well-conserved 3′ part of *chuA* (probe-1). Generation, labeling, and detection of the *chuA*-specific probe as well as the hybridization procedure were performed as described above for Northern blot analysis. Oligonucleotides derived from the dissimilar upstream regions were used to analyze the distribution of the different 5′ flanking regions. Probe-2 (derived from strain 536 [Fig. 3]) (5′-TGA ATT ATC AGA AAT ATT CGG CAA TTT TAC GGG ATA TAT ACG CTA ATA GCT TCC CGT GGT GAT ATC TAA TCA-3′) and probe-3 (derived from the strain EDL933 [Fig. 3]) (5′-CGA GTT ATC AGG CAA TTT CAT GGG ATA TAA ACG C-3′) were purchased from ARK Scientific GmbH (Darmstadt, Germany). The probes were labeled with digoxigenin using the DIG Oligonucleotide 3′-End Labeling kit (Roche, Mannheim, Germany). Prehybridization and hybridization were carried out in high-SDS hybridization buffer at 30°C for 4 h and overnight, respectively. The filters were washed twice for 10 min at room temperature in 2× SSC–0.1% SDS. Hybridized oligonucleotides were detected using the DIG Luminescent Detection kit (Roche) following the standard protocol provided by the manufacturer. The results of the hybridization experiments are summarized in Table 1.

The probe specific for the 3′ end of the *chuA* gene (probe-1) hybridized with numerous intestinal and all extraintestinal pathogenic *E. coli* strains. However, the *chuA*-specific probe hybridized to two distinct bands: to a larger DNA fragment (~12 kb) in case of the extraintestinal and some of the enteropathogenic *E. coli* (EPEC) strains, whereas in EHEC O157, enteroinvasive *E. coli* (EIEC) and some other EPEC strains the *chuA* probe hybridized to a smaller fragment (~11 kb). In correspondence with former investigations, none of the tested non-O157-EHEC, EAggEC, and enterotoxigenic *E. coli* representatives carried *chuA* (41). The oligonucleotide specific for the *chuA* upstream region of strain 536 (probe-2) hybridized to all strains that carried *chuA* on the 12-kb fragment, while that which originated from the O157:H7 strain EDL933 (probe-3) hybridized to the 11-kb fragment, suggesting that two distinct variants of the *chuA* determinant, which show differences in their flanking sequences, exist. The existence of these two variants and their patterned distribution among different pathogroups provides further evidence for the clonality of *E. coli* pathogens. Whether the differences in the *chuA* upstream regions have any influence on the regulation of *chuA* expression still needs to be clarified.

Nucleotide sequence accession number. The nucleotide sequence of the *E. coli* strain 536-specific *chuA* gene has been deposited in the GenBank database (accession number AF280396).

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