Molecular Cloning, Expression, and Regulation in *Escherichia coli* K-12 of a Chromosome-Mediated Aerobactin Iron Transport System from a Human Invasive Isolate of *E. coli* K1

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We have cloned chromosomal genes determining the aerobactin iron transport system from the *Escherichia coli* K1 strain VW187. Mapping and hybridization experiments showed that the VW187 aerobactin region was identical to that of the plasmid ColV-K30. However, in the *E. coli* K-12 background, the biosynthesis of both siderophore and ferric aerobactin receptor encoded by the VW187-derived recombinant plasmids was not repressed by iron to the same extent found when a recombinant plasmid derived from pColV-K30 was used. RNA-DNA dot-blot hybridization experiments demonstrated that the aerobactin-specific mRNA synthesized by the VW187-derived clones was not iron regulated in *E. coli* K-12. In contrast, the synthesis of aerobactin and its receptor in strain VW187 was completely repressed by iron regardless of whether the recombinant plasmids originated from VW187 or pColV-K30. Similar results were obtained with gene fusions in which a promoterless *lac* operon was placed under the control of aerobactin promoter regions of either chromosome- or plasmid-mediated aerobactin systems. DNA sequencing of the chromosomal aerobactin promoter region showed changes in bases located immediately upstream to the –35 region compared with the corresponding region in pColV-K30, which is known to be part of the binding site for the Fur repressor protein.

The aerobactin iron uptake system is a virulence attribute of pathogenic strains of *Escherichia coli* responsible for septicemic infections in human and domestic animals (10, 43). This system was initially described as encoded by ColV-type plasmids (4, 41, 43), and its genetic organization has been elucidated in the past few years (4–6, 9, 12, 13, 15, 16, 22, 31, 35, 42).

We have recently reported that in the majority of the *E. coli* K1 strains causing septicemia and meningitis in human neonates (38, 39) as well as in enteroinvasive strains of *E. coli* (27), the aerobactin system is located on the chromosome. We now report the molecular cloning of the chromosomal genes encoding the aerobactin system in the *E. coli* K1 strain VW187, and we present evidence that the cloned VW187 aerobactin genes and those of pColV-K30 are regulated differently by iron in *E. coli* K-12.

MATERIALS AND METHODS

Materials, bacterial strains, and plasmids. Chemicals and antibiotics used in this study were from Sigma Chemical Co., St. Louis, Mo. Antibiotics were used in the following final concentrations: tetracycline, 20 µg/ml; ampicillin, 100 µg/ml; kanamycin, 30 µg/ml; chloramphenicol, 30 µg/ml; streptomycin, 100 µg/ml; and spectinomycin, 100 µg/ml. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and used as suggested by the supplier.

The properties of the *E. coli* strains and plasmids used in this study are described in Table 1. Strain MV103 is a *lacZ* mutant of strain VW187 isolated after chemical mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Miller (29). Since strain MV103 could not be transformed

with plasmid DNA, we isolated a *galE* mutant by selection for resistance to 2-deoxygalactose as described by Nnalue and Stocker (30). This mutant, designated MV104, was rough and could be transformed with plasmid DNA.

Aerobactin system. Soluble hydroxamate concentrations in supernatants of Luria broth cultures grown under iron-rich (20 µM Fe₃Cl) or iron-limiting (200 µM α,α'-dipyridyl) conditions were determined by the colorimetric method of Csaky (11). To facilitate comparison of the results, the OD₅₄₀ values representing the hydroxamate content of supernatants were corrected by the cell mass as determined by turbidity (OD₆₆₀). Aerobactin production was demonstrated by a cross-feeding bioassay carried out as described elsewhere, with LG1522 as an indicator strain (9, 38). To investigate whether the production of aerobactin was repressed by iron, filtered supernatants from cultures grown overnight at 37°C in L broth containing either 200 µM α,α'-dipyridyl or 20 µM FeCl₃ were used to cross-feed strain LG1522. Prior to the bioassay, supernatants obtained from cells grown with iron were brought up to 200 µM α,α'-dipyridyl to chelate Fe³⁺ and therefore rule out false-positive results. The diameter of the growth halos was measured and used as an indication of relative siderophore production. The presence of the aerobactin receptor was determined by the cloacin DF13 sensitivity test (22). This bacteriocin, produced by *Enterobacter cloacae* DF13, recognizes the ferric aerobactin outer membrane receptor (40). To test for a functional iron transport system, the utilization of exogenously added aerobactin by cells grown under iron stress was determined as described previously (39).

Recombinant DNA methods. Total DNA from strain VW187, isolated by the clear lysis method (19), was cleaved with *Hind*III under conditions in which the cleavage reaction was not complete, giving partially digested DNA fragments. These fragments were centrifuged in a 5 to 25% sucrose gradient in an SW50.1 rotor (Beckman) at 40,000 rpm for 2 h, and fragments of between 15 and 30 kilobase pairs (kb) were

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype, phenotype, or comment ^a	Source or reference
<i>E. coli</i>		
BC3	JK149, <i>cir rpsL</i>	J. Konisky
C2110	<i>gyrA polA rha his</i>	24
HB101	<i>hsdS serA ara proA lacY galK rspL xyl mtl supE</i>	8
LG1522	<i>ara azi fepA lac leu mtl proC rpsL supE tonA tsx thi</i> (pColV-K30 <i>iuc</i>)	9
LE392	<i>hsdS supE supF lacY galK metB trpR</i>	26
H1290	<i>aroB malT tsx thi cys fur-74 Δ(lac-pro)</i>	K. Hantke
TB1	<i>Δ(lac-pro) strA ara thi</i> (F' <i>traD36 proAB lacI^qZM15</i>)	Laboratory stocks
VW187	Clinical isolate, prototrophic, O7:K1	38
MV103	VW187, <i>lacZ</i>	This study
MV104	MV103, <i>gal</i>	This study
Plasmids		
pBRG29	Cm ^r Ap ^r , Tn9 insertion in pBR322 (7), source for the IS1 probe	D. Berg
pHP45Ω	Ap ^r Spc ^r Sm ^r	33
pTL25	Ap ^r , <i>lacZ⁺Y⁺A⁺</i> , high-copy-number vector	25
pFZY1	Ap ^r , <i>lacZ⁺Y⁺A⁺</i> , single-copy-number vector	21
pMH15	Cm ^r , cloned <i>fur</i> gene in high-copy-number vector pACYC184	18
pABN5	Ap ^r , source for the aerobactin siderophore probe	4
pABN6	Ap ^r , source for the aerobactin receptor probe	4
pJHCP3	Cm ^r , source for the REPII probe	31
pJHCP36	Km ^r , source for the REPI probe	32
pJHCV8	Tc ^r , aerobactin genes from pColV-K30 cloned in pVK102 (20)	38
pJHCV24	Tc ^r , cosmid carrying <i>Hind</i> III fragments harboring the aerobactin genes and flanking regions of strain VW187 cloned in pVK102 (20)	This study
pJHCV25	Tc ^r , cosmid carrying <i>Hind</i> III fragments harboring the aerobactin genes and flanking regions of strain VW187 cloned in pVK102 (20)	This study
pJHCV44	Ap ^r , 7.5-kb <i>Hind</i> III fragment from pJHCV24 cloned into pBR322 (7)	This study
pJHCV50	pTL25 with a 3.4-kb <i>Hind</i> III- <i>Bam</i> HI fragment from pJHCV8 carrying the pColV-K30 aerobactin promoter region	This study
pJHCV52	pTL25 with a 3.4-kb <i>Hind</i> III- <i>Bam</i> HI fragment from pJHCV53 carrying the VW187 aerobactin promoter region	This study
pJHCV53	Tc ^r , 10.5-kb <i>Hind</i> III fragment harboring the VW187 aerobactin system genes cloned in pVK102 (20)	This study
pJHCV53-E	pJHCV53 with the Ω fragment (33) inserted in the <i>Eco</i> RI site, cloacin resistance	This study
pJHCV74	0.7-kb <i>Hind</i> III- <i>Sal</i> I fragment harboring the VW187 aerobactin promoter region from pJHCV53 cloned in pUC18	This study
pJHCV83	2.3-kb <i>Sal</i> I fragment harboring the VW187 aerobactin promoter region from pJHCV53 cloned in pFZY1	This study
pJHCV84	2.3-kb <i>Sal</i> I fragment harboring the pColV-K30 aerobactin promoter region from pJHCV8 cloned in pFZY1	This study

^a *iuc*, Mutation in the aerobactin system that impairs siderophore biosynthesis. Probes are shown in Fig. 1.

pooled and precipitated with ethanol. The pellet was suspended in deionized water and ligated to *Hind*III-cleaved pVK102 DNA by using T4 DNA ligase at a molar ratio of vector ends to target of 1:20. The reaction mixture for ligation was packaged in vitro into bacteriophage lambda heads with a commercial extract (Promega Biotech Inc., Madison, Wis.), and the lambda phage particles were transduced into *E. coli* K-12 strain LE392.

Recombinant plasmids generated by cosmid cloning or other cloning procedures were obtained by an alkaline lysis method (26). When needed, plasmid DNA was further purified by centrifugation in cesium chloride-ethidium bromide (38). Electrophoresis of plasmid DNA was performed as described previously (38). Transformations were carried out by the CaCl₂ method (26).

Agarose gels containing plasmid DNA were transferred to nitrocellulose filters by the method of Southern as described in Maniatis et al. (26). The restriction endonuclease DNA fragments used to prepare probes (Table 1 and Fig. 1B) were purified from agarose gels as described previously (3) and labeled with [³²P]ATP by nick translation (26). The probes specific for the internal and flanking regions of the aerobactin

system are described in the legends to Fig. 1 and Table 1. In some experiments, a 0.7-kb *Eco*RI-*Pst*I fragment of pBR322 containing part of the β-lactamase gene was used as a probe to normalize for variations in the recovery of RNA. The conditions for DNA-DNA hybridization of bacterial colonies and Southern blots were as previously reported (38, 39).

Isolation of total RNA and RNA-DNA hybridizations. Total RNA was isolated by the cesium chloride method (14) from cells grown in M9 minimal medium supplemented with either 50 μM α,α'-dipyridyl or 50 μM FeCl₃. Dot-blot RNA-DNA hybridizations were carried out essentially as described by Roberts et al. (35) with 20 and 40 μg of RNA per dot. The amount of RNA hybridizing to the probe was quantitated by excising the portion of the nitrocellulose filter corresponding to the dot and determining bound radioactivity by liquid scintillation counting.

Outer membrane proteins. Sarkosyl-extracted outer membrane proteins were prepared as described previously (1, 39) from cells grown under high- or limited-iron conditions similar to those used for total RNA preparation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described elsewhere (1). Outer

FIG. 1. Genetic and physical map of the aerobactin region and flanking sequences from the chromosome of strain VW187 (A) and from plasmid ColV-K30 (B). (A) Dashed line shows the location of the aerobactin region in VW187. Solid lines indicate the boundaries of DNA fragments from the VW187 aerobactin region cloned in pVK102 (pJHCV24, pJHCV25, pJHCV53, and pJHCV53-E), pBR322 (pJHCV44), pTL25 (pJHCV52), pUC18 (pJHCV74), and pFZY1 (pJHCV83). pJHCV53-E is pJHCV53 with the Ω fragment inserted in the *EcoRI* site. Dotted line for pJHCV83 represents a portion of vector DNA from pJHCV53 that was cloned in pFZY1 together with the 0.7-kb *HindIII-SalI* fragment harboring the aerobactin promoter. (B) pColV-K30 replication regions REPI and REPII (31), and the region harboring the genes for the biosynthesis of siderophore (AEROBACTIN) and receptor (74K). Solid boxes show the location of *IS1* insertion sequences. The restriction endonuclease fragments used as radiolabeled probes were: probe 1, 3.4-kb *HindIII-BamHI* fragment (siderophore probe); probe 2, 2.9-kb *KpnI* fragment (receptor probe); probe 3, 6.7-kb *HindIII-EcoRI* fragment; probe 4, 7.6-kb *BamHI* fragment (REPII probe); and probe 5, 6-kb *EcoRI* fragment with a deletion eliminating the *IS1* insertion sequence (REPI probe [32]). Restriction endonucleases sites: B, *BamHI*; E, *EcoRI*; H, *HindIII*; K, *KpnI*; S, *SalI*. The complete *KpnI* and *SalI* map of the VW187 aerobactin region is not shown.

membrane proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose paper (0.45- μ m pore size; BA85; Schleicher & Schuell, Inc., Keene, N.H.) as described by Towbin et al. (37). After electrophoresis, the Western blots (immunoblots) were incubated with antiserum raised against the 74-kilodalton (kDa) aerobactin receptor protein encoded by pColV-K30, washed, incubated again with horseradish peroxidase-conjugated staphylococcal protein A, and finally stained with H_2O_2 and horseradish peroxidase color development reagent (Bio-Rad Laboratories). The antiserum was raised in 6-month-old New Zealand rabbits by injecting crushed polyacrylamide gel fragments containing the 74-kDa aerobactin receptor protein (27).

Determination of β -galactosidase activity. β -Galactosidase activity was determined as described by Miller (29). M9 minimal medium cultures were grown to an OD_{600} of 0.2 and then divided into two samples which were supplemented with either 200 μ M α,α' -dipyridyl or 50 μ M $FeCl_3$. Incubation was continued for 3 h, and samples were taken at various time points. Lysis of cells was carried out by the procedure of Putnam and Koch (34).

DNA sequencing. The DNA sequence of the VW187 aero-

bactin promoter region was determined by the terminator method of Sanger et al. (36) with [^{35}S]dATP (Amersham) for labeling. The 0.7-kb *HindIII-SalI* fragment from pJHCV53 containing the aerobactin promoter was cloned in pUC18 (pJHCV74; Table 1 and Fig. 1A). pJHCV74 DNA was denatured by treatment with 0.2 M NaOH for 10 min, and single strands were precipitated with ethanol. Both strands were used as templates for sequencing essentially as described previously (17). IBI/Pustell Sequence Database Manager software (International Biotechnologies, Inc., New Haven, Conn.) was used to analyze the sequence data. Only the DNA sequence of the aerobactin promoter region is shown and compared with the corresponding sequence of the pColV-K30 aerobactin promoter P1 reported by Bindeireif and Neilands (6).

Analysis of polypeptide gene products. An in vitro-coupled transcription-translation system (Amersham) was used as specified by the manufacturer. Polypeptides were labeled with [3H]leucine and separated by electrophoresis in a 15% polyacrylamide gel. The gel was fixed in 30% methanol-7% acetic acid, dried, and fluorographed as described before (1).

RESULTS

Cloning of the aerobactin genes from strain VW187. The chromosomal genes encoding the aerobactin system in strain VW187 were cloned into the *E. coli* K-12 strain LE392 as described in Materials and Methods. A gene library of about 900 clones was screened by colony hybridization. Two independent colonies harboring the recombinant plasmids pJHCV24 and pJHCV25 (Fig. 1A) hybridized to the aerobactin-specific probe (Fig. 1B, probe 3). Bacteria carrying either of these two plasmids produced a hydroxamate compound, cross-fed the indicator strain LG1522, and utilized exogenously supplied aerobactin. These cells were also susceptible to cloacin DF13, a bacteriocin that recognizes the aerobactin receptor (40). Similar results with HB101 cells transformed with either pJHCV24 or pJHCV25 DNA confirmed that these recombinant plasmids encoded a functionally intact aerobactin system.

pJHCV24 and pJHCV25 DNAs digested with *Hind*III yielded, in addition to the 23-kb *Hind*III fragment of vector DNA, fragments of 10.5, 4.2, 1.2, and 0.8 kb common to both plasmids (Fig. 1A). pJHCV24 carried an additional 7.5-kb *Hind*III fragment that was absent in pJHCV25, whereas pJHCV25 harbored a 2-kb *Hind*III fragment that was absent in pJHCV24. The relative position of each of these *Hind*III fragments was determined by restriction endonuclease mapping with a variety of enzymes (Fig. 1A). Hybridization experiments with both siderophore and receptor probes (Fig. 1B, probes 1 and 2) localized the aerobactin genes within the 10.5-kb *Hind*III fragment (Fig. 1A, and data not shown); therefore, this fragment was subcloned in pVK102, resulting in pJHCV53. The restriction endonuclease map of pJHCV53 (Fig. 1A) was compared with that of pJHCV8, a recombinant plasmid harboring the pColV-K30 aerobactin genes (Fig. 1B and Table 1). Both plasmids had an identical restriction endonuclease map in the regions corresponding to the siderophore biosynthesis and receptor genes (Fig. 1A and B).

In contrast, mapping analysis of the cosmids pJHCV24 and pJHCV25 revealed that the regions flanking the VW187 aerobactin genes (Fig. 1A) differed from the corresponding regions in pColV-K30 (Fig. 1B). Two *Hind*III fragments of 1.8 and 7.5 kb were found upstream from the chromosomal aerobactin genes (Fig. 1A), whereas the corresponding region in pColV-K30 consisted of a single 8.6-kb *Hind*III fragment (Fig. 1B). Moreover, three *Hind*III fragments of 4.5, 0.8, and 2 kb were located downstream of the chromosomal aerobactin genes instead of the 62-kb *Hind*III fragment present in the corresponding region of pColV-K30 (Fig. 1B) (31, 42). The aerobactin genes in pColV-K30 are flanked by two copies of *IS*I insertion sequences (23, 28, 31) and also by two distinct replication regions, REPI and REPII (31). No hybridization was detected in either pJHCV24 or pJHCV25 with either *IS*I- or REPII-specific probes, whereas the REPI probe showed homology with the 7.5-kb *Hind*III fragment in pJHCV24 (data not shown). However, pJHCV44, a recombinant plasmid containing the 7.5-kb fragment from pJHCV24 cloned in pBR322 (Fig. 1A), did not replicate when transformed into the polymerase I-deficient strain C2110. Hence, this experiment demonstrated that the 7.5-kb *Hind*III fragment does not possess a functional replication region.

Expression of the aerobactin receptor outer membrane protein encoded by the cloned VW187 aerobactin genes. The pColV-K30 aerobactin receptor is an outer membrane protein with an apparent molecular mass of 74 kDa (4, 9, 22). To

FIG. 2. Iron-regulated outer membrane proteins encoded by recombinant plasmids harboring the chromosomal aerobactin system. Coomassie blue-stained polyacrylamide gel showing the electrophoretic profiles of outer membrane proteins (30 μ g of protein per lane) obtained from strain BC3 with no plasmid (A and E) or carrying pJHCV8 (B and F), pJHCV24 (C and G), or pJHCV53 (D and H). Cells were grown with 200 μ M α,α' -dipyridyl (A, B, C, D) or 20 μ M FeCl_3 (E, F, G, H). Lanes m, Molecular mass markers. KD, Kilodaltons.

investigate the expression of a similar receptor by the cloned VW187 aerobactin genes, strain BC3 was transformed with pJHCV24, pJHCV53, and pJHCV8 as a positive control. Strain BC3 has a mutation in the *cir* locus and consequently does not synthesize the colicin Ia outer membrane receptor, which comigrates with the aerobactin receptor in SDS-polyacrylamide gels. pJHCV8, pJHCV24, and pJHCV53 encoded a 74-kDa outer membrane protein (Fig. 2, lanes B, C, D, F, G, and H) that was absent in cell envelopes from strain BC3 carrying the vector pVK102 (Fig. 2, lanes A and E). The 74-kDa outer membrane protein encoded by either pJHCV24 or pJHCV53 cross-reacted immunologically with antiserum raised against the 74-kDa outer membrane protein encoded by pJHCV8, as revealed by immunoblotting experiments (Fig. 3, lanes C to F). Also, the wild-type strain VW187 expressed a 74-kDa outer membrane protein immunologically related to the pColV-K30 aerobactin receptor (Fig. 3, lanes A and B). To assess whether the 74-kDa outer membrane protein encoded by clones derived from strain VW187 was involved in the transport of ferric aerobactin, we constructed a mutant by inserting a 2-kb restriction endonuclease fragment carrying the *Spc*^r *Sm*^r gene (Ω fragment [33]) in the *Eco*RI site of pJHCV53 (Fig. 1A). Cells carrying this plasmid, designated pJHCV53-E, did not express the 74-kDa outer membrane protein (Fig. 3) and were unable to utilize exogenously supplied aerobactin. As expected, these cells were also no longer susceptible to cloacin DF13.

Regulation by iron of synthesis of the 74-kDa outer membrane protein and siderophore encoded by clones derived from strain VW187. Synthesis of the 74-kDa outer membrane

FIG. 3. Western blot analysis of the aerobactin receptor outer membrane protein encoded by recombinant plasmids in *E. coli* K-12. Outer membrane proteins separated by SDS-PAGE were electrotransferred to nitrocellulose paper and reacted with rabbit antiserum raised against the 74-kDa aerobactin receptor encoded by pJHCV8 as described in Materials and Methods. Lanes contained VW187 wild-type (A and B) or strain BC3 carrying pJHCV8 (C and D), pJHCV53 (E and F), or pJHCV53-E (G and H). Cells were grown with 200 μ M α,α' -dipyridyl (lanes A, C, E, G) or 20 μ M FeCl_3 (lanes B, D, F, H).

TABLE 2. Aerobactin production mediated by recombinant plasmids in *E. coli* K-12 and VW187 strains as determined by cross-feeding bioassays

Strain	Plasmid ^a	Derivation of DNA insert	Siderophore activity (diam of halo in mm) of supernatants from cells grown with:	
			α, α' -Dipyridyl ^b (200 μ M)	FeCl ₃ (20 μ M)
<i>E. coli</i> K-12 ^c	pVK102	No insert	—	—
	pJHCv8	pColV-K30	+ (1.8)	—
	pJHCv53	VW187	+ (1.5)	+ (1.4)
VW187	pVK102	No insert	+ (1.6)	—
	pJHCv8	pColV-K30	+ (1.7)	—
	pJHCv53	VW187	+ (1.5)	—

^a Plasmid integrity was confirmed by restriction endonuclease analysis.^b Supernatants were brought to a final concentration of 200 μ M α, α' -dipyridyl before being used for the bioassays.^c Same results were obtained with strains BC3, HB101, and LE392 (see Table 1).

protein in the wild-type strain VW187 and in BC3 cells carrying pJHCv8 was reduced to comparable low levels under iron-rich growth conditions (Fig. 2, lane F, and Fig. 3, lanes B and D). In contrast, synthesis of the 74-kDa protein by BC3 cells carrying either pJHCv53 or pJHCv24 was not reduced by iron to the same extent (Fig. 2, lanes G and H, and Fig. 3, lanes E and F). Analysis by densitometry of the gel shown in Fig. 2 revealed that the amount of the 74-kDa polypeptide encoded by either pJHCv24 or pJHCv53 under iron-rich conditions (Fig. 2, lanes G and H) was two- to threefold higher than the amount of the pJHCv8-encoded 74-kDa polypeptide (lane F). Also, we examined whether aerobactin production mediated by either pJHCv8 or the VW187 clones pJHCv24, pJHCv25, and pJHCv53 was subject to the same differences found in the regulation of synthesis of the 74-kDa protein. BC3 cells carrying each of these plasmids were grown under iron stress and iron-rich conditions, and the presence of aerobactin in their supernatants was determined by a cross-feeding bioassay as described in Materials and Methods. BC3 carrying the VW187 clones produced aerobactin in both iron-limiting and iron-rich conditions of growth, whereas BC3 carrying pJHCv8 produced aerobactin only under iron stress (Table 2). Similar results were obtained with other strains of *E. coli* K-12, such as HB101 and LE392. In addition, total hydroxamate production relative to cell mass was determined to estimate the amount of aerobactin produced under iron-rich and iron-limiting conditions by cells carrying pJHCv8 and pJHCv24. The results showed a 19.5-fold reduction by iron in the amount of hydroxamates produced by cells carrying pJHCv8, whereas the amount of hydroxamates produced by cells containing pJHCv24 was reduced only 3.8-fold. Since the genes encoding the pColV-K30 and the VW187 aerobactin systems were cloned into the same cloning vectors, the differences in their iron regulation cannot be explained merely by a gene dosage effect. In addition, these differences may not be due to the participation of vector sequences, since the orientation of the inserted DNA in pJHCv8, pJHCv24, and pJHCv53 was the same.

To investigate whether the difference found in iron regulation of the cloned VW187 aerobactin system in *E. coli* K-12 was related to the level of transcription of aerobactin-specific mRNA, we carried out RNA-DNA dot-blot hybridization experiments with the siderophore probe (Fig. 1B, probe 1) as described in Materials and Methods. Preliminary experiments indicated that the amount of probe hybridizing to the aerobactin-specific mRNA was in excess of complementary sequences contained within a range of 20 to 40 μ g of total RNA per dot. Transcripts of the aerobactin biosynthe-

sis region in pJHCv8 were approximately 10- to 20-fold less abundant in total RNA prepared from BC3(pJHCv8) grown in the presence of iron (50 μ M FeCl₃) than in total RNA obtained from BC3(pJHCv8) grown in the presence of an iron chelator (200 μ M α, α' -dipyridyl) (Table 3). In contrast, the amount of aerobactin-specific mRNA mediated by pJHCv53 did not vary considerably in BC3(pJHCv53) grown in the presence of iron or dipyridyl. Preparations of RNA from BC3 cells carrying the vector pVK102 showed virtually no hybridization to the probe. These experiments therefore demonstrate that the synthesis of aerobactin-specific mRNA by the VW187 clones in *E. coli* K-12 is not iron repressible. However, the levels of aerobactin-specific mRNA detected in BC3(pJHCv53) were six- to eightfold less abundant than those of mRNA synthesized by BC3(pJHCv8). This finding was reproducible and consistently observed with various RNA preparations.

Next, we investigated the iron regulation of the aerobactin system in the wild-type cell background. pJHCv24, pJHCv25, pJHCv53, and pJHCv8 were mobilized by triparental matings to a Nal^r derivative of strain VW187 by using the helper plasmid pRK2013 (Table 1). The physical integrity of these plasmids in strain VW187 was confirmed by restriction endonuclease mapping to rule out the possibility of recombination with chromosomal aerobactin sequences (data not shown). Both aerobactin production and synthesis of the 74-kDa outer membrane receptor protein were induced only under conditions of iron limitation in all cases (Table 2, Fig. 4). Thus, these experiments demonstrate that expression of the aerobactin mediated by the VW187 clones is iron regulated in strain VW187 but is poorly regulated in the *E. coli* K-12 background. In contrast, the pColV-K30-derived aerobactin system did not show any difference in regulation by iron in either VW187 or *E. coli* K-12 strains.

Gene fusion experiments. To quantitate the differences in the iron regulation of both aerobactin systems, we constructed gene fusions to a promoterless *lac* operon by using

TABLE 3. RNA-DNA hybridization analysis of transcription of plasmid and chromosomal aerobactin systems^a

Addition to medium (μ M)	Hybridization to RNA (mean cpm $\pm$ SD) ^b		
	BC3(pJHCv8)	BC3(pJHCv53)	BC3(pVK102)
α, α' -Dipyridyl (200)	1,201 $\pm$ 4	150 $\pm$ 11	51 $\pm$ 19
FeCl ₃ (20)	65 $\pm$ 17	150 $\pm$ 12	55 $\pm$ 18

^a Dot-blot hybridizations were carried out with probe 3 (Fig. 1) as described in Materials and Methods with 20 μ g of total RNA per dot.^b Determined by liquid scintillation counting.

FIG. 4. Western blot analysis of the aerobactin receptor outer membrane protein encoded by recombinant plasmids in strain VW187. Outer membrane proteins were processed as described in the legend to Fig. 3. Lanes: A and B, VW187 carrying pJHCV8; C and D, strain VW187 carrying pJHCV53; E and F, strain VW187 carrying no plasmid; G, strain BC3 with no plasmid. Cells were grown with 200 μ M α, α' -dipyridyl (lanes A, C, E, G) or 20 μ M FeCl_3 (lanes B, D, F).

plasmid pTL25 (Table 1) (25). A 3.4-kb *HindIII*-*Bam*HI fragment containing the aerobactin promoter region and downstream sequences from either pJHCV8 or pJHCV53 was cloned in the corresponding sites of the polylinker in pTL25, generating pJHCV50 and pJHCV52, respectively. The time course of β -galactosidase activity in M9 cultures of *E. coli* TB1 (Table 1) harboring either pJHCV50 or pJHCV52 was determined before and after induction and repression. In TB1(pJHCV50), the synthesis of β -galactosidase was induced under iron stress up to about 4,000 U (Fig. 5a). Repression by iron caused synthesis to reach a basal level of 300 U after 60 min, which corresponded to 13-fold less β -galactosidase compared with the level detected after induction. In the case of TB1(pJHCV52), the β -galactosidase activity detected under iron stress was 7,000 U (Fig. 5a), higher than that detected in TB1(pJHCV50). Under high iron, the enzyme activity of TB1(pJHCV52) increased up to about 2,500 U at 60 min and decreased slowly to 1,500 U upon continuing growth. The β -galactosidase activities were on average three- to fivefold lower than the enzyme activities detected after induction. A control experiment with TB1 carrying the vector pTL25 showed a basal β -galactosidase activity of about 250 U, similar to the value reported previously (25). DNA-RNA dot-blot hybridization experiments with probe 3 (Fig. 1) were also carried out with total RNA preparations obtained from TB1 cells containing

pJHCV50 and pJHCV52. In this case, hybridizations with a β -lactamase probe fragment (see Materials and Methods) were included as an internal control for RNA recovery. The amount of aerobactin-specific mRNA detected correlated with the level of expression found with pJHCV50 and pJHCV52 under iron-rich and iron-limiting conditions (data not shown).

The fusion plasmids were transformed into MV104, a *lacZ gal* mutant of VW187, and the time course of β -galactosidase activity under iron-rich and iron-limiting conditions of growth was also determined. In control experiments, MV104 with and without pTL25 exhibited enzyme activity of 250 and 30 U, respectively. MV104 harboring either pJHCV50 or pJHCV52 showed comparable levels of enzyme activity after induction and repression (Fig. 5b). In both cases, repression by iron resulted in approximately 10-fold less β -galactosidase compared with the level detected under iron limitation.

To evaluate the effect of gene dosage in the regulation by iron of both aerobactin systems, similar gene fusions were constructed with the single-copy-number vector pFZY1 (Table 1) (21). Plasmids pJHCV83 and pJHCV84 contained a 2.3-kb *SalI* fragment from pJHCV53 and pJHCV8, respectively, cloned in the appropriate orientation to generate a transcriptional fusion with the *lac* operon sequences located downstream from the cloning site (Fig. 1A) (21). Figure 5c shows that TB1 cells containing either of the two plasmids exhibited a similar degree of iron-regulated β -galactosidase activity. These results demonstrate that the gene dosage of the aerobactin promoter influences iron regulation in *E. coli* K-12 strains.

The gene fusion experiments also showed that in all cases the recombinant clones derived from the VW187 aerobactin system expressed higher β -galactosidase activity than pColV-K30-derived clones. The higher level of β -galactosidase expression determined by the chromosomal clones did not depend on differences in cell growth, since viable-cell counts were found to be comparable for TB1 cells containing either pJHCV50 or pJHCV52 when grown under the same iron-rich or iron-limiting conditions. Furthermore, this result

FIG. 5. Iron regulation of the synthesis of β -galactosidase fusion protein. (a and b) Strains TB1 and MV104 (VW187 *lacZ gal*) carrying either pJHCV50 ($\times$) or pJHCV52 ($\circ$). (c) Strain TB1 carrying either pJHCV83 ($\circ$) or pJHCV84 ($\times$). Cells were grown in M9 minimal medium, divided into two samples at time zero, and induced by adding α, α' -dipyridyl (200 μ M) or repressed by the addition of 20 μ M FeCl_3 as described in Results. The time course of β -galactosidase activity (in units of enzyme per A_{600} unit of cell growth) after induction (—) and repression (----) at time zero is shown. Note that the scale of β -galactosidase activity in panel c is different.

FIG. 6. In vitro-coupled transcription-translation. Autoradiograph showing the translation products of the 3.4-kb *Hind*III-*Bam*HI DNA fragments of pJHCV50 (A) and pJHCV52 (B). Arrows point to a 33-kDa polypeptide corresponding to the *iutB* gene product that is barely visible in the photograph. *, 30-kDa polypeptide encoded by the *Km^r* gene fragment of pUC4K that was included in all the reaction mixes as an internal standard. Lane M, molecular mass markers.

appeared to be independent of the Fur protein, since it was observed in *fur* mutants and also in experiments carried out with TB1 cells containing either pJHCV50 or pJHCV52 in conjunction with pMH15 (Table 1, and data not shown). This plasmid overexpresses the Fur protein and is compatible with pJHCV50 and pJHCV52 (18). In addition, these differences did not depend on the gene dosage, since they were also present in the experiments carried out with plasmids pJHCV83 and pJHCV84 (Fig. 5c).

Thus, we speculated that the VW187 aerobactin genes are better able to express the protein components of this operon. To test this hypothesis, we carried out an in vitro-coupled

transcription-translation experiment with the 3.4-kb *Hind*III-*Bam*HI fragments isolated from pJHCV50 and pJHCV52 used as DNA templates. These fragments carry the aerobactin promoter and the complete structural genes for the first two enzymes involved in aerobactin biosynthesis. Each of these fragments was mixed with the same amount of the 1.4-kb *Eco*RI *Km^r* gene fragment isolated from pUC4K, which served as an internal standard. The autoradiograph (Fig. 6) was analyzed by densitometry, and the result showed that the DNA fragment isolated from pJHCV52 expressed two- to threefold more of the 62- and 33-kDa polypeptides relative to the 30-kDa *Km^r* gene product than did pJHCV50 (Fig. 6, lanes A and B). This in vitro experiment therefore confirms the in vivo results obtained with the gene fusions, indicating that the aerobactin genes derived from the VW187 chromosome are better expressed than the corresponding genes from pColV-K30.

DNA sequence of the VW187 aerobactin promoter region. The different iron regulation of the cloned VW187 aerobactin genes in *E. coli* K-12 than of the pColV-K30 aerobactin system could be associated with changes in the nucleotide sequence of the VW187 aerobactin promoter region. Therefore, the 0.7-kb *Hind*III-*Sal*I fragment of pJHCV53 containing the aerobactin promoter was subcloned in pUC18, resulting in pJHCV74 (Table 1 and Fig. 1A). Both DNA strands of the insert in pJHCV74 were sequenced as described in Materials and Methods. The DNA sequence of this 0.7-kb *Hind*III-*Sal*I fragment was compared with the reported DNA sequence of the pColV-K30 aerobactin promoter region (6), which includes 412 nucleotides from the *Hind*III end to the presumed initiation codon of the first structural gene of the aerobactin operon. The DNA sequences of the pColV-K30 and VW187 aerobactin promoter regions were sufficiently homologous to be aligned (Fig. 7). Small changes were detected in the region upstream from the aerobactin promoter P1, encompassing the promoter P2 sequences and its upstream region up to the *Hind*III site (data not shown). The -35 and -10 boxes of the VW187 P1 promoter were identical to the corresponding regions in

FIG. 7. Comparison of the DNA sequences of the aerobactin promoter regions from strain VW187 (A) and pColV-K30 (B). Only the region corresponding to the promoter P1 is shown. The organization of the pColV-K30 aerobactin promoter region (B) and the boundaries of the primary binding site for the Fur repressor were determined previously by other investigators (6, 13). The -35 and -10 regions are indicated, as is the ribosome-binding site (SD). The first transcribed nucleotide in panel B is indicated (*) (6). Bases in panel A not present in panel B are indicated in lowercase letters; a dash symbolizes the absence in panel A of a T found in panel B. ●, Shared 5'-TCATT-3' sequences. Thin and thick arrows indicate the first and second overlapping dyad symmetries, respectively (6, 13).

pColV-K30 (Fig. 7). The presumed ribosome-binding site in VW187 (5'-GGGAGCTGT-3') differed in one nucleotide from its counterpart in pColV-K30 (5'-AGGAGCTGT-3', Fig. 7). In the P1 promoter of pColV-K30, there is a region spanning from 7 base pairs (bp) upstream of the -35 region to the first base pair of its -10 sequence which was defined as the primary binding site for the Fur repressor protein (13). This region contains two overlapping symmetry dyads, which have a common sequence (5'-TCATT-3') repeated three times through the Fur binding site, with a uniform spacing of 3 bp (Fig. 7B) (13). In VW187, the nucleotides upstream from the -35 region (5'-TATTGT-3') differed from those in the same position in pColV-K30 (5'-TTCTCA-3'), which resulted in the absence of the first dyad symmetry (Fig. 7). Moreover, the sequence ACATT was present in VW187 instead of the sequence TCATT of pColV-K30, and the spacing was 5 bases instead of 3 nucleotides (Fig. 7A). Thus, the comparison of the nucleotide sequences of the VW187 and pColV-K30 P1 aerobactin promoters showed that they differed in the binding site for the Fur repressor protein and also in the putative ribosome-binding site region.

DISCUSSION

The aerobactin iron transport system was initially described as encoded by ColV-type plasmids (4, 41, 43). However, a chromosome-mediated aerobactin system is frequently found among *E. coli* K1 strains of human origin (38, 39). To characterize the chromosome-determined aerobactin system and compare it with the plasmid system, we cloned and expressed in *E. coli* K-12 the chromosomal aerobactin genes from the *E. coli* K1 strain VW187. Hybridization and restriction endonuclease mapping experiments showed that the physical map of the VW187 chromosomal aerobactin system was virtually identical to that of the system encoded by the prototypic plasmid ColV-K30. This is in agreement with our previous results, which indicated a high degree of conservation between the VW187 and pColV-K30 aerobactin systems (38).

Despite the similarities in the restriction endonuclease map between VW187 and pColV-K30 aerobactin systems, the synthesis of both siderophore and receptor encoded by recombinant clones derived from strain VW187 was not repressed by iron in *E. coli* K-12 to the same extent as observed with a clone derived from pColV-K30. These differences cannot be explained by gene dosage effects, since the same low-copy-number cloning vectors were used. The participation of vector sequences in determining these differences is also unlikely, since DNA inserts in the subclones derived from either pColV-K30(pJHCV8) or VW187 (pJHCV53) were in the same orientation. In addition, expression of the cloned pColV-K30 and VW187 aerobactin systems in the VW187 strain background was regulated at the same level. Therefore, our results demonstrate that the difference in the iron regulation of both systems is only found in the *E. coli* K-12 background, and this finding rules out the possibility that a mutation in the VW187 cloned genes occurred during cloning manipulations.

The analysis of aerobactin-specific RNA transcripts carried out in this work showed that aerobactin mRNA synthesized in *E. coli* K-12 by clones derived from strain VW187 was not iron repressible. In the case of pColV-K30, the aerobactin system is transcriptionally regulated by a repression mechanism mediated by the Fur protein (13, 18), a repressor for several iron uptake systems in *E. coli* strains (2, 18). The Fur protein impairs the initiation of transcription

at the level of the iron-regulated aerobactin promoter by binding to an operator region that overlaps with promoter sequences (2, 13). Therefore, the results presented in this work suggest that the Fur protein cannot repress aerobactin-specific mRNA synthesis by the cloned VW187 aerobactin operon in the *E. coli* K-12 cell background. Furthermore, the total amount of aerobactin-specific mRNA determined by VW187-derived clones was six- to eightfold lower than that synthesized by pColV-K30-derived plasmids. This may indicate that the aerobactin-specific mRNA mediated by the chromosomal genes is more unstable than that synthesized by the ColV-K30 plasmid system.

Gene fusion experiments were carried out to better characterize the differences found in expression of the chromosomal aerobactin system and the pColV-K30 system. The β -galactosidase activity in an *E. coli* K-12 strain carrying a plasmid with a transcriptional fusion between VW187 aerobactin sequences and the *lac* operon was not significantly repressed under iron-rich conditions. However, this effect proved to be gene dosage dependent, since fusions performed with a single-copy-number vector exhibited similar iron-regulated β -galactosidase activity regardless of whether the fusion was derived from pColV-K30 or VW187 aerobactin sequences. Furthermore, the cloned chromosomal aerobactin system showed overall higher β -galactosidase activity in vivo as well as increased amounts of aerobactin-specific polypeptides in vitro.

From the previous results, we expected differences in the aerobactin promoter regions of the VW187 chromosomal system and the pColV-K30 system. Consequently, the nucleotide sequence of the VW187 aerobactin promoter region was determined and compared with the corresponding region of pColV-K30 (6). This comparison revealed that the -10 and -35 boxes of the VW187 aerobactin promoter sequences were very similar to those of the corresponding regions in pColV-K30, except for a different base composition of nucleotides located immediately upstream from the -35 box. The nucleotide changes in the VW187 aerobactin promoter resulted in the absence of one of the two dyad symmetries found to be part of the Fur-binding site in the corresponding region of pColV-K30 (6, 13). This may account for the low level of iron regulation of the VW187 clones observed in the *E. coli* K-12 background. However, the iron regulation of the chromosomal aerobactin system in *E. coli* K-12 did not seem to be affected by these changes if the aerobactin genes were present at a low gene dosage. This conclusion is supported by the results of *lac* fusion experiments with a single-copy-number cloning vector. Since both pColV-K30- and VW187-derived clones showed the same level of iron regulation in the VW187 strain background regardless of their gene dosage, it is likely that the Fur protein synthesized by the strain VW187 binds efficiently to promoter regions from both pColV-K30 and VW187 aerobactin systems despite the differences in the Fur-binding site, whereas the Fur of K-12 strains may prefer the plasmid-encoded aerobactin system. Our results with *fur* mutants of *E. coli* K-12 and with strains in which the *fur* gene (cloned in a high-copy-number plasmid) was a coresident of the chromosomal aerobactin system clone confirmed that an excess of the K-12 Fur protein did not affect expression of the VW187 system. It is somewhat surprising that differently effective Fur operators exist in *E. coli* for the VW187 and K-12 systems in the light of quite high variations in the Fur-binding consensus sequence and the activity of the *E. coli* Fur in distantly related organisms (13, 18).

Analysis of the DNA sequence of the VW187 aerobactin

promoter also showed that there was a single base pair change in the putative ribosome-binding site region (a G instead of an A in the pColV-K30 aerobactin promoter). We have not tested directly whether this change is reflected in different translation of the VW187 aerobactin structural gene products. However, *in vivo* and *in vitro* expression of the VW187 aerobactin genes was markedly higher than that of the homologous genes from pColV-K30. These results suggest that the VW187 aerobactin genes are more efficiently translated than those from pColV-K30.

Another feature of the chromosomal and pColV-K30 aerobactin systems is the differences in the regions flanking the aerobactin genes. In this plasmid, the aerobactin genes are flanked by two copies of *IS1* insertion sequences (23, 28, 31) in inverted orientation (31) and also by two distinct replication regions, REPI and REPII (31). Further studies in this laboratory have demonstrated that the *IS1* and REPI regions are conserved in all the ColV aerobactin plasmids examined (42). In contrast, our results demonstrate that this is not the case in the VW187 chromosomal aerobactin genes. Furthermore, the regions flanking chromosomal aerobactin genes in other strains of *E. coli* K1 belonging to various clonal groups varied from one clone to another, whereas the regions internal to the aerobactin system were conserved (M. A. Valvano and J. H. Crosa, manuscript in preparation). These results suggest that the chromosomal aerobactin genes in *E. coli* K1 strains from various clonal groups may have arisen from insertion at different locations in the bacterial chromosome.

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