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High-Affinity Iron Uptake Systems Present in *Erwinia carotovora* subsp. *carotovora* Include the Hydroxamate Siderophore Aerobactin

CAROL A. ISHIMARU† AND JOYCE E. LOPER*

United States Department of Agriculture, Agricultural Research Service, Horticultural Crops Research Laboratory, 3420 N.W. Orchard Avenue, and Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97330

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The phytopathogenic bacterium *Erwinia carotovora* subsp. *carotovora* W3C105 produced the hydroxamate siderophore aerobactin under iron-limiting conditions. A survey of 22 diverse strains of *E. carotovora* revealed that strain W3C105 alone produced aerobactin. The ferric-aerobactin receptor of strain W3C105 was an 80-kDa protein, identified by immunoblots of Sarkosyl-soluble proteins obtained from *E. carotovora* cells grown in iron-depleted medium and probed with antiserum raised against the 74-kDa ferric-aerobactin receptor encoded by the pColV-K30 plasmid of *Escherichia coli*. Genes determining aerobactin biosynthesis and uptake were localized to an 11.3-kb *Eco*RI-*Hin*dIII chromosomal fragment of strain W3C105. A 10-kb subclone of the fragment conferred on *E. coli* DH5α both aerobactin biosynthesis and uptake, determined by cloacin DF13 sensitivity, the presence of the 80-kDa receptor protein, and iron-independent growth of *E. coli* clones. The aerobactin biosynthesis genes of *E. carotovora* W3C105 hybridized to those of the pColV-K30 plasmid of *E. coli*, but the restriction patterns of the aerobactin regions of *E. coli* and *E. carotovora* differed. Although the aerobactin region of enteric bacteria is commonly flanked by IS*1*-like sequences, IS*1* sequences were not detected in the genomic DNA or the cloned aerobactin region of *E. carotovora*. *E. coli* DH5α cells harboring cloned aerobactin biosynthesis genes from *E. carotovora* W3C105 produced greater quantities of aerobactin and the 80-kDa ferric-aerobactin receptor when grown in iron-limited than in iron-replete medium. Strain W3C105 grew on an iron-limited medium, whereas derivatives that lacked a functional aerobactin iron acquisition system did not grow on the medium. These results provide evidence for the occurrence and heterogeneity of aerobactin as a high-affinity iron uptake system of both clinical and phytopathogenic species of the *Enterobacteriaceae*. Although future studies may reveal a role for aerobactin in the virulence or ecology of strain W3C105, a functional aerobactin iron acquisition system is not necessary for the pathogenicity of *E. carotovora*.

Iron is an essential element for living organisms by virtue of its two valences that act as cofactors in various oxidative-reductive enzymatic reactions. Iron is abundant on the earth's crust, yet in aerobic environments at neutral pH, it exists as insoluble iron oxides, which are largely unavailable biologically. Thus, most organisms have systems for the specific chelation and regulated transport of iron into the cell. With some exceptions, microorganisms use siderophores and corresponding membrane receptors for iron acquisition. Siderophores are low-molecular-weight, Fe(III)-specific ligands that are produced by organisms as iron-scavenging agents when available forms of iron are limited (39). Many enterobacteria synthesize catechol siderophores, such as enterobactin (also called enterochelin), or hydroxamate siderophores, such as aerobactin (41). Aerobactin was isolated first from cultures of *Aerobacter aerogenes* (22). Other bacterial species within the family *Enterobacteriaceae*, including *Shigella flexneri* (40), *Enterobacter cloacae* (14, 56), and *Escherichia coli* (12, 61), also synthesize aerobactin. Biosynthesis of aerobactin is one of several virulence factors in invasive *E. coli* strains (41, 60, 61), enabling bacterial proliferation in the iron-deficient intercellular environment of mammalian tissues.

Erwinia carotovora subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* are phytopathogenic members of the *Enterobacteriaceae* that cause soft-rot diseases of potato (*Solanum tuberosum* L.). These phytopathogens are prevalent in agricultural soils and in the plant rhizosphere (43). Soilborne pathogens, such as *E. carotovora*, presumably experience iron limitation at the oxygen levels and pH ranges present in many agricultural soils (10). A preliminary report indicates that *E. carotovora* subsp. *carotovora* produces an uncharacterized catechol siderophore (34), but a role for siderophores in the virulence or ecology of this phytopathogen has not been defined. The related bacterium *Erwinia chrysanthemi* produces a catechol siderophore, chrysobactin (44), which contributes to the systemic virulence of this phytopathogen (18). Similarly, an uncharacterized hydroxamate siderophore is a virulence factor of *Erwinia amylovora* (55), the causal agent of fire blight disease.

Erwinia soft rot of potato can be controlled biologically by application of antagonistic *Pseudomonas* spp. to potato seed pieces prior to planting (13, 26, 63) or to tubers prior to storage (13). Biological control of potato seed piece decay is thought to be determined by siderophore-mediated iron competition between *Pseudomonas* spp. and *E. carotovora* (26, 63). It is hypothesized that the fluorescent siderophores, termed pyoverdines (also pyoverdins or pseudobactins), produced by *Pseudomonas* spp. deplete the pathogens' microenvironment of available iron by sequestering ferric ions as ferric-pyoverdine complexes, which are utilized exclusively by *Pseudomonas* spp. (32, 35). Exchange of

* Corresponding author.
† Present address: Department of Plant Pathology and Weed Science, Colorado State University, Fort Collins, CO 80523.

Fe(III) between pyoverdines and *Erwinia* siderophores is expected to determine siderophore-mediated iron competition between *Pseudomonas* spp. and *E. carotovora* (35). Thus, characterization of the siderophores produced by *E. carotovora* is critical to the hypothesis that pyoverdines produced by *Pseudomonas* spp. limit the levels of iron available to *E. carotovora*. Nevertheless, the siderophore production and utilization systems of *E. carotovora* are virtually unknown.

Our studies focused on characterization of the iron acquisition systems of *E. carotovora* and elucidation of the importance of siderophores in the ecology, pathogenicity, and biological control of soft-rot erwiniae. The importance of siderophores in the biological control of soft-rot diseases and in the virulence of a closely related *Erwinia* sp. prompted our investigations of siderophore production by *E. carotovora*. In this report, we present evidence for hydroxamate siderophore production by a strain of *E. carotovora* subsp. *carotovora*. We identify the hydroxamate as aerobactin and characterize the genes encoding aerobactin and the ferric-aerobactin receptor. This is the first report of aerobactin production by a plant-pathogenic bacterium.

(An abstract of this research has been published [25a].)

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used are listed in Table 1. Twenty-two field isolates of *E. carotovora* (Table 2) were selected for this study on the basis of previous evaluation as target pathogens for biological control by antagonistic pseudomonads (63), distinct serotype groupings (23, 45), or diverse hosts or environmental sources of isolation. *E. coli* and *E. carotovora* were cultured routinely on Luria-Bertani (LB) medium (48) at 37 or 27°C, respectively. The growth rates of strains of *E. carotovora* were determined by the change in OD_{640} of cultures grown with shaking at 27°C in LB broth. In some cloning experiments, LB agar was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/ml; International Biotechnologies, Inc.) and isopropyl-β-D-thiogalactopyranoside (100 μg/ml; Sigma) for screening of transformants. Siderophores were produced in M9 medium (38) or Tris-minimal salts medium (TMS) (50). Where specified, M9 and TMS were supplemented with casamino acids (0.3%), tryptophan (0.003%), thiamine (0.002%), and glucose (0.2%) (CM9 and CTMS). Stock solutions of casamino acids were extracted with 8-hydroxyquinoline and chloroform (4) to remove contaminating iron. Crystal violet-pectate (CVP) agar (16) and pectate agar (5) were the selective media for culture of *E. carotovora*.

Antibiotics (Sigma) were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 20 μg/ml, except when different concentrations are specified.

Detection of siderophore production. Siderophore production was detected by observation of orange halos surrounding test strains grown on CAS agar (49). Catechol production was detected from supernatants of cultures grown for 24 to 48 h in CM9 or CTMS by the methods of Arnow (1) or Rioux et al. (47). Hydroxamate(s) in culture supernatants was detected by the methods of Atkin et al. (2) and Csáky (15).

Aerobactin bioassay. Aerobactin production by bacterial strains was detected by crossfeeding of the indicator strain, *E. coli* LG1522 (12, 61). *E. coli* LG1522, which does not produce aerobactin or enterobactin but has the ferric-aerobactin receptor, cannot grow under iron-limiting conditions unless it is provided with iron in the form of ferric-aerobactin. Molten TMS containing 150 μM 2,2'-dipyridyl was seeded with approximately 10^6 CFU of strain LG1522 per ml. Strains to be evaluated for aerobactin production were spotted onto the surface of solidified, seeded TMS medium and incubated at 27°C. After 24 to 48 h, a halo of growth of the indicator strain surrounded the aerobactin-producing colonies. *E. coli* strains RWB18 and LG1522-1, which cannot utilize aerobactin, were indicator strains serving as negative controls in the bioassay.

Purification and characterization of the hydroxamate produced by W3C105. Hydroxamates were isolated, by the method of Gibson and Magrath (22), from 3-liter cultures grown in CM9 medium for 24 to 48 h. Hydroxamates were separated on cellulose thin-layer chromatography (TLC) plates (Analtech MN300) in a butanol-acetic acid-water (60:15:25) solvent system and visualized by spraying the plates with ferric chloride (0.4% [wt/vol] in 40 mM HCl). Samples were prepared for ^1H nuclear magnetic resonance (NMR) studies on a Bruker WM400 instrument by repeated exchanges in deuterium oxide. Assignment of the methylene protons from hydroxylysine was made by comparison with the spectrum of the unmodified amino acid (62).

Nucleic acid isolation and hybridization. Plasmids from *E. coli* and *E. carotovora* were isolated by an alkaline lysis procedure (48) and purified by ethidium bromide-cesium chloride density gradient centrifugation. For isolation of genomic DNA, cells were lysed with sodium lauryl sulfate (SDS), treated with proteinase K, and extracted with hexadecyltrimethylammonium bromide in chloroform (48) prior to standard phenol-chloroform extraction and ethanol precipitation. Electrophoresis was done in 0.5 to 0.7% agarose gels with Tris-phosphate-EDTA (TPE) buffer (48). Standard procedures for ligations, alkaline phosphatase treatments, restriction endonuclease digestions, and transformation procedures were used (48). For Southern hybridizations, DNA was transferred to nylon membranes (Nytran; Schleicher & Schuell) according to the manufacturer's directions.

The plasmid pABN1 (7) (Fig. 1B), which contains the aerobactin biosynthesis (*iuc*) and ferric-aerobactin receptor (*iut*) genes of the pColV-K30 plasmid of *E. coli*, was the source of DNA fragments used as nucleic acid probes. Probes (Fig. 1B) were as follows: (i) the 7.0-kb *Hin*dIII-*Eco*RI fragment of pABN1 (*iuc-iut* probe), which contained genes for aerobactin biosynthesis (*iucABCD*) and the truncated gene for the ferric-aerobactin receptor (*iutA*); (ii) the 1.8-kb *Pvu*II-*Kpn*I fragment (*iut* gene probe); (iii) the 2-kb *Pvu*II fragment (IS*1* probe); and (iv) the 2-kb *Ava*I fragment (*iucBC* probe). Restriction fragments used as probes were purified from agarose gels by adsorption and elution from NA-45 DEAE membranes, according to the recommendations of the manufacturer (Schleicher & Schuell). Nucleic acid probes, prepared by nick translation of isolated fragments with biotin-ATP (Bethesda Research Laboratories), were used at concentrations of 0.2 μg/ml of hybridization solution. Hybridizations were visualized following development with a nonradioactive nucleic acid detection kit (Blu GENE R; Bethesda Research Laboratories). Radioactive probes, labeled by nick translation or random primer extension with [^{32}P]dCTP according to the recommendations of the manufacturer (Bethesda Research Laboratories), were used in some experiments. Hybridization conditions were moderately stringent (42°C, 50% formamide and 0.16× SSC) and were followed by washes at 55°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Genomic library construction. Purified genomic DNA from

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Relevant characteristics[a]	Source or reference
Escherichia coli			
BN3040 Nal^r	*entA cir*	Ent$^-$ Nal^r	25
DH5α	F$^-$ *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1* φ80*dlacZ*Δ M15 λ$^-$	Ent$^+$	Bethesda Research Laboratories
LG1315	*ara entA lac leu mtl proC rpsL supE thi tonA trpE xyl* (pColV-K30)	Ent$^-$ Iuc$^+$ Iut$^+$	60
LG1522	*ara azi fepA lac leu mtl proC rpsL supE tonA tsx thi iuc* (pColV-K30*iuc*)	FepA$^-$ Iuc$^-$ Iut$^+$	60
LG1522-1	As LG1522, but cured of pColV-K30*iuc*	FepA$^-$ Iuc$^-$ Iut$^-$	L. Crosa, Oregon Health Sciences University, Portland
RWB18	*entA fepA*	Ent$^-$ FepA$^-$ Iuc$^-$ Iut$^-$	59
VW187	Clinical isolate	Iuc$^+$ Iut$^+$ Ent$^+$	52
Enterobacter cloacae			
EK33	Clinical isolate	Iuc$^+$ Iut$^+$ Ent$^+$	14
DF13	Cloacin DF13 producer		56
Erwinia carotovora subsp. *carotovora*			
W3C105	Field isolate	Iuc$^+$ Iut$^+$	63
JL11,178	Derivative of W3C105, Δ(*iuc*)	Iuc$^-$ Iut$^+$	This study
JL11,182	Derivative of W3C105, Δ(*iuc-iut*)	Iuc$^-$ Iut$^-$	This study
JL11,215	Derivative of W3C105, Δ(*iuc*)::*nptI-sacB-sacR*	Iuc$^-$ Iut$^+$ Km^r, sucrose sensitive	This study
Plasmids			
pABN1	*iucABCD*$^+$ *iutA*$^+$, IS*1* in pPlac	Iuc$^+$ Iut$^+$ Ap^r	7
pUC8	ColE1 replicon	Ap^r LacZ$^+$	57
pLAFR3	*cos*, *inc*P1 replicon, polylinker of pUC8	Tc^r LacZ$^+$ Mob$^+$	51
pRK2013	Mobilizing plasmid	Tra$^+$ Km^r	20
pUM24	pUC4K derivative containing *nptI-sacB-sacR* cartridge	Km^r Ap^r, sucrose sensitive	46
pJEL1530	28-kb fragment from W3C105 cloned into pLAFR3	Iuc$^+$ Iut$^-$ Tc^r	This study
pJEL1531	21-kb fragment from W3C105 cloned into pLAFR3	Iuc$^+$ Iut$^+$ Tc^r	This study
pJEL1532	22-kb fragment from W3C105 cloned into pLAFR3	Iuc$^+$ Iut$^+$ Tc^r	This study
pJEL1533	16-kb fragment from W3C105 cloned into pLAFR3	Iuc$^+$ Iut$^+$ Tc^r	This study
pJEL1534	26-kb fragment from W3C105 cloned into pLAFR3	Iut$^+$ Iut$^+$ Tc^r	This study
pJEL1540	6.2-kb *Eco*RI fragment of pJEL1530 cloned into pUC8	Iuc$^+$ Iut$^-$ Ap^r	This study
pJEL1545	10-kb *Eco*RI fragment of pJEL1534 cloned in pUC8	Iuc$^+$ Iut$^+$ Ap^r	This study
pJEL1551	11.3-kb *Eco*RI-*Hin*dIII fragment of pJEL1533 cloned into pUC8	Iuc$^+$ Iut$^+$ Ap^r	This study
pJEL1745	5.4-kb *Bgl*II-*Eco*RI fragment of pJEL1545 cloned into pUC8	Iut$^+$ Ap^r	This study
pJEL1814	*Eco*RI-*Bam*HI fragment of pJEL1551 with Δ(*iuc*)::*nptI-sacB-sacR* cloned into pLAFR3	Iuc$^-$ Iut$^+$ Tc^r Km^r	This study
pJEL1816	*Eco*RI-*Hin*dIII fragment of pJEL1551 with Δ(*iuc*) cloned into pLAFR3	Iuc$^-$ Iut$^+$ Tc^r	This study
pJEL1963	*Eco*RI-*Hin*dIII fragment of pJEL1551 with Δ(*iuc-iut*) cloned into pLAFR3	Iuc$^-$ Iuc$^-$ Tc^r	This study

[a] Ent$^+$ and Ent$^-$, enterobactin producer or nonproducer, respectively; Nal^r, nalidixic acid resistant; Iuc$^+$ and Iuc$^-$ aerobactin producer or nonproducer, respectively; Iut$^+$ and Iut$^-$, possesses or lacks, respectively, the outer membrane receptor for ferric aerobactin; FepA$^-$, lacks outer membrane receptor protein for ferric enterobactin; Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Tc^r, tetracycline resistant; LacZ$^+$, β-galactosidase activity; Mob$^+$, mobilizable plasmid; Tra$^+$, self-transmissible plasmid.

E. carotovora W3C105 was digested partially with *Sau*3AI, and fragments were separated in a 0.4% agarose gel. Fragments in the range of 15 to 30 kb were eluted from the gel and ligated into the dephosphorylated *Bam*HI site of pLAFR3, a broad-host-range cosmid cloning vector (51). Ligated DNA was packaged into phage heads and tails (Gigapack Plus; Stratagene); *E. coli* DH5α was transduced with packaged DNA, and transductants were selected on LB with tetracycline.

Detection of aerobactin utilization and cloacin sensitivity. Utilization of ferric-aerobactin was evaluated by a previously described bioassay (54). Solid CTMS was amended with 150 to 200 μM 2,2′-dipyridyl and inoculated with about 10^3 CFU of a test strain per ml. Ten microliters of aerobactin (120 to 560 μM), purified from *E. coli* LG1315, was spotted onto a sterile paper disk placed on the surface of the seeded CTMS agar. Alternatively, 2 μl of a culture of LG1315 was spotted onto the surface of seeded CTMS agar. Halos of growth surrounding the paper disk or colony of LG1315 were indicative of aerobactin utilization.

The presence of a ferric-aerobactin receptor was also demonstrated by measuring sensitivity to cloacin DF13, a bacteriocin that recognizes the ferric-aerobactin receptor protein (6, 56). Strains were tested for sensitivity to crude preparations of cloacin DF13 obtained from filtrates of cultures of *Enterobacter cloacae* DF13, which were treated with mitomycin (1 μg/ml), as described previously (12). Cultures to be tested for cloacin DF13 sensitivity were grown overnight in CTMS and adjusted to a uniform density of 0.1 OD_{640}. Ten microliters of the adjusted suspension was

TABLE 2. Field isolates of *E. carotovora*

Strain	Serogroup	Origin	Source or reference	Siderophore production[a]	Aerobactin production[b]
E. carotovora subsp. *carotovora*					
W3C105	XXXIX	Potato, North Dakota	63	+++	+
cc101	III	Potato	23	+	−
cc102	V	Potato, Montana	23	+	−
cc103	XL	Potato, North Dakota	23	++	−
cc104	XV	Potato, Montana	23	+	−
cc106	XI	Potato, Montana	23	+	−
cc108	XXXVI	Potato, Montana	23	+	−
cc110	XXXVIII	Potato, Oregon	23	+	−
cc501	XXIX	Potato, Oregon	23	+	−
cc505	XXVII	Potato, Oregon	23	+	−
SCRI-193		Soil, Scotland	24	+	−
SR319	XXIX	Soil, Wisconsin	A. Kelman	+	−
cc303	XXXVII	Soil, Oregon	23	++	−
cc306	XXXIII	Soil, Oregon	23	+	−
274-1-2		Water, Colorado	M.Powelson	+	−
JL1128		Artichoke, California	M.Powelson	++	−
JL1131		Onion, Oregon	M.Powelson	+	−
JL1132		Broccoli, Oregon	M.Powelson	+	−
JL1133		Lettuce, California	M.Powelson	+	−
JL1134		Broccoli, Oregon	M.Powelson	+	−
E. carotovora subsp. *atroseptica*					
W3C37		Potato, Washington	63	+	−
SCRI-1043		Potato, Scotland	24	+	−

[a] Radius of orange halo produced in CAS agar: +++, >0.5 cm; ++, 0.2 to 0.5 cm; +, <0.2 cm.
[b] Aerobactin production based on hydroxamate production (2) and crossfeeding of *E. coli* LG1522 (61); +, aerobactin production; −, no aerobactin production.

added to 10 ml of nutrient broth (Difco Laboratories) containing 5% (wt/vol) molten agar. The molten-agar suspension was poured on the surface of nutrient agar in petri plates. Ten microliters of a cloacin DF13 preparation was spotted on the center of the cooled agar surface. Plates were incubated at 37°C for 18 h and observed for clear zones of growth inhibition surrounding the cloacin DF13 preparation.

Analysis of outer membrane proteins and Western immunoblots. Outer membrane proteins were prepared as described before (54) with the following modifications. Cells were grown overnight in 5 ml of CTMS, amended with 20 μM $FeCl_3$ or unamended, and transferred to 50 ml of CTMS amended with the same concentration of $FeCl_3$. After 24 h, cells were harvested, resuspended in 11 ml of 10 mM Tris-hydrochloride–0.3% NaCl (pH 8.0), and disrupted by sonication, and cellular debris was removed. Membranes were pelleted by centrifugation, and Sarkosyl-soluble proteins, containing predominantly outer membrane proteins, were separated on 10% polyacrylamide–SDS gels. Proteins were visualized by staining with Coomassie blue. Western blots of Sarkosyl-soluble proteins probed with antiserum raised against the 74-kDa outer membrane receptor protein from pColV-K30 were developed with an alkaline phosphatase kit from Vector Laboratories. Antiserum to the aerobactin receptor protein from *E. coli* LG1315 was a gift from L. Crosa, Oregon Health Sciences University, Portland.

Derivation of Iuc⁻ and Iuc⁻ Iut⁻ strains of *E. carotovora*. The marker exchange-eviction mutagenesis technique of Ried and Collmer (46) was used to construct directed, unmarked mutations in *E. carotovora* W3C105. The *nptI-sacB-sacR* cartridge, which is carried on a 3.8-kb *Bam*HI fragment, confers on *E. carotovora* kanamycin resistance, due to *nptI*, and sucrose sensitivity, due to the production of levan sucrase by *sacB*. *E. carotovora* grew on 925 agar medium (30), a minimal medium containing 10% sucrose as a sole carbon source, whereas cells that contained the *sacB* gene did not grow on this medium. Exchange recombination events between unstable recombinant plasmids and the chromosome resulting in insertion of the cartridge into the bacterial genome were selected on LB with kanamycin; those resulting in eviction of the cartridge from the genome were selected on medium 925 containing 10% sucrose. In preliminary experiments, pLAFR3 (51) was not stably maintained in *E. carotovora*; it was lost from 90% of the cells of W3C105 after 3 days of culture in the absence of tetracycline selection. Therefore, it seemed likely that genomic fragments cloned into pLAFR3 and rescued by homologous recombination into the genome of W3C105 could be detected. Mobilization of pLAFR3 and derivatives into W3C105 was accomplished by triparental matings with DH5α(pRK2013) (20) as a helper. Transconjugants (*E. carotovora* harboring pLAFR3 derivatives) were selected on CVP or pectate agar amended with tetracycline (60 μg/ml). To obtain strains that had lost pLAFR3 or derivatives, transconjugants were grown at 27°C with shaking in 200 ml of LB broth in the absence of tetracycline. After 10 to 24 hours, 0.1 ml of culture was transferred to 200 ml of fresh LB medium. After five successive transfers, pLAFR3 derivatives were generally lost from greater than 99% of the bacterial cells.

Conditions of iron-limited growth. Strains of *E. carotovora* were grown overnight at 27°C with shaking in TMS broth amended with 0.1 μM $FeCl_3$. Five microliters of the culture was spotted onto the surface of TMS agar supplemented with 150 μM 2,2′-dipyridyl.

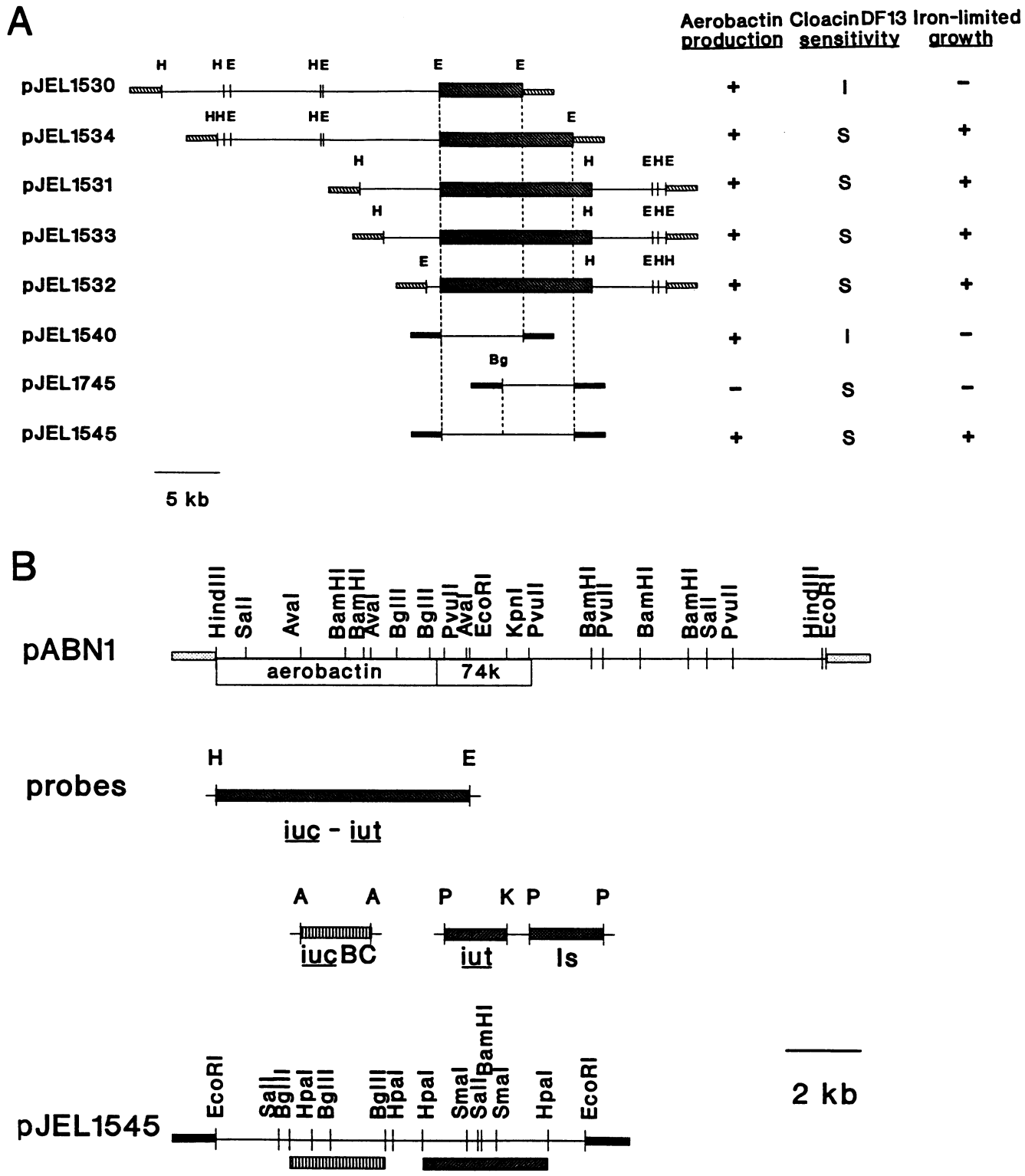

FIG. 1. Identification of aerobactin genes of *E. carotovora* subsp. *carotovora* W3C105. (A) Restriction maps and phenotypes conferred by selected cosmids and plasmids containing aerobactin genes from *E. carotovora* W3C105. Thick diagonally shaded bars within the map indicate regions that hybridize to the *iuc-iut* genes of pColV-K30, shown in panel B. Bars at the end of each construct indicate vector DNA (hatched bars, pLAFR3; solid bars, pUC8). Aerobactin production was detected by a crossfeeding bioassay with *E. coli* LG1522 as an indicator. Cloacin DF13 sensitivity (S) or insensitivity (I) was determined by bioassay of *E. coli* BN3040 Nal^r or DH5α harboring the indicated plasmids. Iron-limited growth of BN3040 Nal^r harboring the indicated plasmids was evaluated on TMS containing 200 μM 2,2′-dipyridyl. (B) DNA probes and restriction maps of aerobactin genes from the pColV-K30 plasmid of *E. coli* (cloned in pABN1) and from *E. carotovora* W3C105 (cloned in pJEL1545). No restriction sites for *Bcl*I, *Sst*I, *Xba*I, *Pvu*II, and *Kpn*I were identified within the 10-kb insert of pJEL1545. *Ava*I sites were not determined. Shaded regions below the map of pJEL1545 indicate restriction fragments that hybridize to probes composed of the *iucBC* or *iut* gene of pColV-K30. Abbreviations: Is, IS*1*; A, *Ava*I; Bg, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II; R, *Eco*RI.

TABLE 3. Proton NMR chemical shift data for aerobactin isolated from *E. coli* LG1315 and from *E. carotovora* subsp. *carotovora* W3C105 in deuterium oxide

Proton assignment	Chemical shift (multiplicity)[a]	
	E. coli LG1315 (pColV-K30)	*E. carotovora* subsp. *carotovora* W3C105
Lys α	4.08 (t)	4.07 (t)
Lys ε	3.54 (t)	3.57 (t)
Citrate CH_2	2.67 (d of q)	2.70 (d of q)
Acetyl CH_3	2.06 (s)	2.06 (s)
Lys β,δ	1.48–1.83 (m)	1.57–1.72 (m)
Lys γ	1.22–1.38 (m)	1.22–1.36 (m)

[a] Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

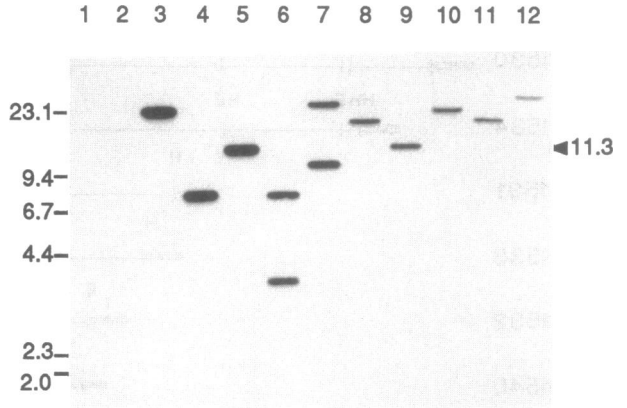

FIG. 2. Southern analysis of genomic DNA from *E. coli* VW187 (lanes 3 to 7) and *E. carotovora* W3C105 (lanes 8 to 12) probed with the *iuc-iut* region of pColV-K30. Also shown are *E. coli* DH5α (lane 1) and *E. carotovora* SR319 (lane 2), which do not produce aerobactin. DNA was digested with *Eco*RI (lanes 1, 2, 3, and 8), *Hin*dIII (lanes 5 and 10), *Bam*HI (lanes 7 and 12), *Eco*RI and *Hin*dIII (lanes 4 and 9), or *Hin*dIII and *Bam*HI (lanes 6 and 11). Sizes are shown in kilobases.

RESULTS

Survey of catechol and hydroxamate production by strains of *E. carotovora*. Each of the 22 field isolates of *E. carotovora* produced catechol in CTMS and an orange halo on CAS agar, indicating the production of a siderophore (Table 2). Large orange halos (0.5 to 1.0 cm from the margin of the colony) were observed in CAS agar surrounding colonies of *E. carotovora* W3C105 (Table 2) and known aerobactin-producing strains, such as *E. coli* LG1315 (data not shown). Small halos (<0.5 cm) surrounded the colonies of other strains of *E. carotovora* or strains of *E. coli* that produced enterobactin but not aerobactin.

Hydroxamate production was detected in supernatants of *E. carotovora* W3C105 but not in those of other strains of *E. carotovora*. Strain W3C105 crossfed the aerobactin indicator *E. coli* LG1522 but not *E. coli* RWB18 or LG1522-1. None of the other *E. carotovora* strains crossfed *E. coli* LG1522 (Table 2).

Purification and characterization of aerobactin produced by *E. carotovora*. Aerobactin, purified from *E. coli* LG1315, and the iron-reactive hydroxamate isolated from *E. carotovora* W3C105 had in common an R_f of 0.58 on TLC plates. No additional hydroxamates were detected in culture supernatants of *E. carotovora* W3C105. The proton NMR spectrum of the hydroxamate from *E. carotovora* W3C105 was consistent with authentic aerobactin (Table 3) and with the published spectrum of aerobactin (22).

Identification and cloning of aerobactin biosynthesis genes of *E. carotovora* W3C105. *E. carotovora* strains were screened by Southern blot analysis for DNA sequences that hybridized to the *iuc-iut* genes of the pColV-K30 plasmid of *E. coli*. Of the 22 strains, only strain W3C105 contained a genomic region that hybridized to the *iuc-iut* probe. A single 11.3-kb *Eco*RI-*Hin*dIII fragment of genomic DNA isolated from strain W3C105 hybridized to the *iuc-iut* genes of *E. coli* (Fig. 2). A cryptic plasmid isolated from *E. carotovora* W3C105 did not hybridize to the *iuc-iut* probe. Other plasmids indigenous to *E. carotovora* W3C105 were not detected. Thus, the aerobactin genes of strain W3C105 appeared to be chromosomal.

To further characterize the aerobactin genes of *E. carotovora* W3C105, a cosmid library was constructed in *E. coli* DH5α, which produces enterobactin but not aerobactin. Five of the 1,050 cosmid clones that constituted the genomic library of *E. carotovora* W3C105 conferred aerobactin production on *E. coli* DH5α, as indicated by crossfeeding of *E. coli* LG1522 and production of large orange halos in CAS agar. Cosmids isolated from these five clones contained a common region that hybridized to the *iuc-iut* probe (Fig. 1A). Three of the five cosmids, pJEL1531, pJEL1532, and pJEL1533, contained an 11.3-kb *Eco*RI-*Hin*dIII fragment that hybridized to the *iuc-iut* probe. The hybridizing *Eco*RI fragments in the other two cosmids were 10 kb (pJEL1534) and 6.2 kb (pJEL1530), presumably due to truncation of the native *Hin*dIII-*Eco*RI fragment of W3C105 by *Sau*3AI partial digestion in construction of the genomic library. The subcloned 6.2-kb *Eco*RI fragment of pJEL1530, 10.0-kb *Eco*RI fragment of pJEL1534, and 11.3-kb *Eco*RI-*Hin*dIII fragment of pJEL1533 each conferred aerobactin biosynthesis on DH5α (Fig. 1A).

Analysis of ferric-aerobactin uptake genes of *E. carotovora*. *E. coli* strains harboring cosmids or subclones containing the 11.3-kb *Eco*RI-*Hin*dIII or 10-kb *Eco*RI fragments that hybridized to the *iuc-iut* probe were sensitive to cloacin DF13 (Fig. 1A). *E. coli* BN3040 Nal^r, which is deficient in enterobactin production, harboring these plasmids also grew on TMS medium containing 200 μM 2,2′-dipyridyl (Fig. 1A) and synthesized an 80-kDa outer membrane protein (Fig. 3) that cross-reacted with antiserum to the 74-kDa ferric-aerobactin receptor from *E. coli* LG1315 (Fig. 4). Strains of *E. coli* BN3040 Nal^r harboring pJEL1530 or pJEL1540, a subclone containing the 6.2-kb *Eco*RI fragment of pJEL1530 that hybridized to the *iuc-iut* probe, were insensitive to cloacin DF13 (Fig. 1A), did not grow on TMS amended with 200 μM 2,2′-dipyridyl (Fig. 1A), and did not synthesize the 80-kDa outer membrane protein (Fig. 3 and 4). Thus, the genes conferring aerobactin production were located within the 6.2-kb *Eco*RI fragment of pJEL1540, while the genes for the ferric-aerobactin receptor were not wholly contained in this fragment. Plasmid pJEL1745 (Fig. 1A), which is composed of a 5.4-kb *Bgl*II-*Eco*RI fragment cloned in pUC8, conferred the ferric-aerobactin receptor, as determined by cloacin DF13 sensitivity, but did not confer aerobactin production to *E. coli*. Strain BN3040 Nal^r harboring both pJEL1530 and pJEL1745 produced aerobactin, was sensitive to cloacin DF13, and grew on TMS amended with 200 μM 2,2′-dipyridyl.

Restriction endonuclease mapping of aerobactin genes of *E. carotovora*. Comparison of the restriction endonuclease maps of the aerobactin production and receptor regions revealed

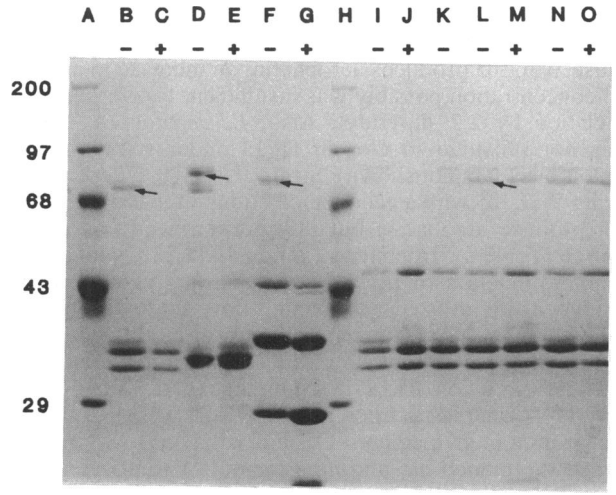

FIG. 3. Profile of Sarkosyl-soluble outer membrane proteins isolated from bacterial cultures grown in a medium amended with 20 μM $FeCl_3$ (+) or unamended (−). Numbers on the left indicate the positions of molecular mass markers (in kilodaltons) in lanes A and H. Proteins were stained with Coomassie blue. Strains tested: *E. coli*(pABN1), lanes B and C; *Enterobacter cloacae* EK33, lanes D and E; *E. carotovora* W3C105, lanes F and G; *E. coli* BN3040 Nal^r containing pLAFR3 (lanes I and J), pJEL1530 (lane K), pJEL1534 (lanes L and M), or pJEL1533 (lanes N and O). Arrows point to the 74-kDa ferric-aerobactin receptor from pColV-K30 (lane B), 85-kDa ferric-aerobactin receptor from *Enterobacter cloacae* EK33 (13) (lane D), and an antigenically related 80-kDa protein made by *E. carotovora* W3C105 (lane F) and clones of *E. coli* BN3040 Nal^r (lanes L, M, N, and O).

substantial differences between *E. coli* and *E. carotovora* (Fig. 1B and 2). Nevertheless, the *iucBC* and *iut* genes of *E. coli* hybridized to the aerobactin region of *E. carotovora*. A single 3.4-kb *Hpa*I fragment on the right side of the cloned aerobactin region of *E. carotovora* hybridized to the *iutA* gene probe (Fig. 1B). Two contiguous *Bgl*II fragments, of 1.1 and 1.5 kb, hybridized to the *iucBC* gene probe. The locations of the *iutA*- and *iucBC*-hybridizing restriction fragments (Fig. 1B) are consistent with the bioassay data, presented above, which indicates that the ferric-aerobactin uptake region is on the right side of the region, as depicted in Fig. 1B, while the aerobactin biosynthesis region is located to the left. Restriction fragments of genomic DNA of W3C105 that hybridized to the *iutA* and *iucBC* gene probes

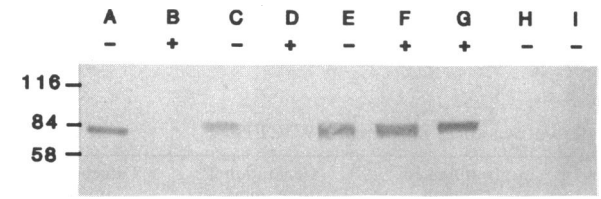

FIG. 4. Western blot of Sarkosyl-soluble outer membrane proteins from bacterial cultures grown in a medium amended with 20 μM $FeCl_3$ (+) or unamended (−) and probed with antiserum to the 74-kDa ferric-aerobactin receptor encoded by pColV-K30. Protein samples: *E. coli* VW187 (lanes A and B); *E. carotovora* W3C105 (lanes C and D); *E. coli* BN3040 Nal^r containing plasmid pJEL1533 (lanes E and F), pJEL1534 (lane G), pJEL1530 (lane H), or pLAFR3 (lane I). The positions of molecular mass markers are indicated on the left (in kilodaltons).

TABLE 4. Aerobactin production by selected clones of *E. coli* containing aerobactin genes from *E. carotovora* W3C105[a]

Plasmid	Aerobactin production (μM)			
	Strain BN3040 Nal^r		Strain DH5α	
	0.1 μM $FeCl_3$	10 μM $FeCl_3$	0.1 μM $FeCl_3$	10 μM $FeCl_3$
pJEL1530	NG	136	186	5
pJEL1531	137	87	257	5
pJEL1532	152	86	327	5
pJEL1533	183	144	353	8
pJEL1534	173	112	308	6

[a] Micromolar equivalents of aerobactin measured by the Csáky assay (15) of supernatants collected from broth cultures grown for 48 h in TMS amended with either 0.1 or 10 μM $FeCl_3$. NG, no growth. Values are the average of three replications, each adjusted for cell density (A_{550}/A_{640}).

were identical in size to the hybridizing fragments of pJEL1545, which contained the cloned 10-kb *Eco*RI fragment of *E. carotovora* (data not shown).

On the pColV-K30 plasmid of *E. coli*, aerobactin genes are flanked by two copies of the IS*1* insertion sequence (31, 37, 42). To determine whether the aerobactin genes of *E. carotovora* W3C105 were also flanked by IS*1* elements, *Eco*RI-cleaved genomic DNA of W3C105 and of the five cosmids conferring aerobactin production (Fig. 1A) were hybridized with the IS*1* probe (Fig. 1B). No hybridization of genomic or cloned aerobactin genes of W3C105 to the IS*1* probe was observed by Southern analysis. Therefore, no evidence for the presence of IS*1* insertion sequences in the proximity of W3C105 aerobactin genes was obtained.

Iron regulation of aerobactin genes of *E. carotovora*. *E. carotovora* W3C105 produces 138 μM hydroxamate in TMS medium containing 0.1 μM $FeCl_3$ but no detectable hydroxamate (<7 μM) in medium containing 10 μM $FeCl_3$. Similarly, the 80-kDa protein, which cross-reacted with antiserum to the ferric-aerobactin receptor protein of *E. coli*, was present in the outer membrane of *E. carotovora* W3C105 cells grown under low iron concentrations but not detected in cells grown in medium amended with 20 μM $FeCl_3$ (Fig. 4). *E. coli* DH5α containing the aerobactin biosynthesis genes of strain W3C105 also produced greater concentrations of aerobactin in an iron-limited medium than in a medium amended with 10 μM $FeCl_3$ (Table 4). In contrast, *E. coli* BN3040 Nal^r harboring the aerobactin biosynthesis and uptake genes of *E. carotovora* produced aerobactin and the 80-kDa receptor protein in iron-replete and iron-depleted media (Table 4, Fig. 3 and 4). These experiments indicate that expression of aerobactin mediated by the W3C105 clones is iron regulated in strain DH5α but is poorly regulated in the *E. coli* BN3040 Nal^r background.

Derivation of Iuc$^-$ and Iuc$^-$ Iut$^-$ mutants of *E. carotovora*. Sequences within the *iuc* or *iuc-iut* region were deleted from the genome of *E. carotovora* by the marker exchange-eviction mutagenesis technique of Ried and Collmer (46). The *nptI-sacB-sacR* cartridge was ligated in place of the deleted *iuc* region of *Bgl*II-digested pJEL1551, which was the *Eco*RI-*Hin*dIII fragment of pJEL1533 (Fig. 1) subcloned into pUC8 (Fig. 5). The *Eco*RI-*Hin*dIII fragment containing the *nptI-sacB-sacR* cartridge and flanking sequences was cloned in the unstable cosmid pLAFR3 to construct pJEL1814 (Fig. 5). *Bgl*II and *Sal*I fragments internal to pJEL1551 were deleted; *Eco*RI-*Hin*dIII fragments containing the deletions were subcloned into pLAFR3 to construct

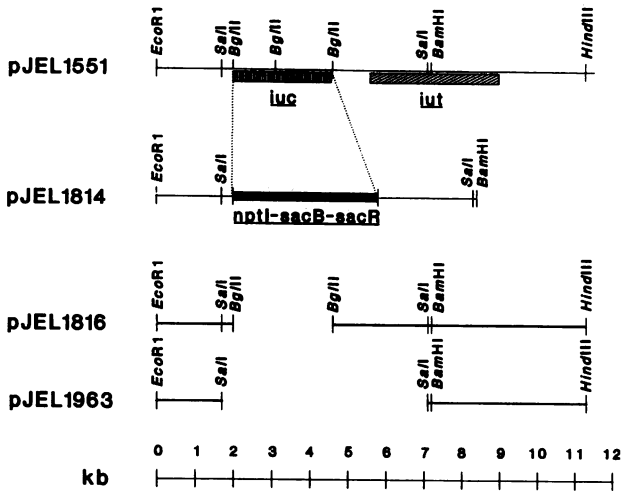

FIG. 5. Restriction map of the deletions in cloned *iuc* and *iut* genes used to construct the Iuc$^-$ and Iuc$^-$ Iut$^-$ mutants of *E. carotovora*. The location of regions that hybridize to the *iucBC* and *iutA* genes, as indicated in Fig. 1, and relevant restriction sites are shown. Dashed lines denote the replacement of sequences with the *nptI-sacB-sacR* cartridge. Deletion derivatives were used to mutate strain W3C105 to JL11,178 and JL11,182 via a series of gene replacements: pJEL1814 (W3C105 to JL11,215), pJEL1816 (JL11,215 to JL11,178), and pJEL1963 (JL11,215 to JL11,182).

unstable plasmids with deletions in the *iuc* (pJEL1816) and *iuc-iut* (pJEL1963) regions, respectively (Fig. 5). Cartridge-containing sequences in pJEL1814 were exchanged into wild-type strain W3C105 to produce JL11,215. The cartridge was then evicted from JL11,215 by exchange substitution of the sequences in pJEL1816 to produce JL11,178. Alternatively, the cartridge was evicted from JL11,215 by exchange substitution of sequences in pJEL1963 to produce JL11,182. Southern analysis of restriction enzyme-digested DNA probed with biotin-labeled pJEL1814 confirmed that the insertions and deletions into the genomic DNA of W3C105 were as shown in Fig. 5 (data not shown).

Characterization of Iuc$^-$ and Iuc$^-$ Iut$^-$ mutants of *E. carotovora*. Definitive evidence for the presence of a ferric-aerobactin receptor in *E. carotovora* W3C105 required analysis of aerobactin-deficient mutants, since attempts to demonstrate uptake in *E. carotovora* directly were unsuccessful. For example, iron deprivation of *E. carotovora* W3C105 on iron-depleted medium (TMS amended with >200 μM 2,2′-dipyridyl) was not consistently alleviated by the addition of up to 500 μM purified aerobactin to the medium. Growth of W3C105 was completely inhibited at 400 μM 2,2′-dipyridyl. We presume that functional catechol siderophore and aerobactin iron uptake systems of strain W3C105 complicated the interpretation of the aerobactin utilization assay. Because W3C105 produces aerobactin, an increase in aerobactin concentration possibly was insufficient to overcome iron chelation by 2,2′-dipyridyl. Also, *E. carotovora* W3C105 was not sensitive to cloacin DF13 under standard assay conditions (data not shown). Strain JL11,178, a derivative of strain W3C105 with a genomic deletion in the *iuc* region, did not produce aerobactin but utilized aerobactin as an iron source (Table 5). In contrast, strain JL11,182, which had a genomic deletion of both the *iuc* and *iut* regions, neither produced nor utilized aerobactin. Both mutants produced catechol, were prototrophic, and grew at a rate comparable to that of the wild-type strain in LB broth medium. Strain W3C105 grew on TMS medium amended with 150 μM dipyridyl, whereas neither JL11,178 nor JL11,182 grew on this iron-limited medium. Cosmid pJEL1534, which contained the cloned *iuc* and *iut* genes of W3C105 (Fig. 1A), restored aerobactin production and utilization and capacity for iron-limited growth to both the Iuc$^-$ and the Iuc$^-$ Iut$^-$ mutants (Table 5). Thus, a functional aerobactin iron acquisition system in *E. carotovora* W3C105 was demonstrated.

DISCUSSION

Siderophore production is a common characteristic of the phytopathogen *E. carotovora*; each of the 22 strains evaluated in this study produced a positive reaction on the universal siderophore medium of Schwyn and Neilands (49) (Table 2) and produced catechol, as has been reported previously for one strain of *E. carotovora* (34). The present study focused on one strain, *E. carotovora* subsp. *carotovora* W3C105, which was unique in having a functional aerobactin iron uptake system, as demonstrated by several lines of evidence. The proton NMR spectrum of the sole hydroxamate produced by strain W3C105 was identical to that of authentic aerobactin (Table 3) and to the published spectrum of aerobactin (22). Hybridization experiments with aerobactin-specific probes from the pColV-K30 plasmid of *E. coli* demonstrated the presence of aerobactin genes in *E. carotovora*. Cloned genes, which encoded the *E. carotovora* aerobactin iron uptake system, conferred aerobactin production on host cells of *E. coli*, as determined by a specific crossfeeding bioassay. Strain W3C105 grew on an iron-limited medium, whereas Iuc$^-$ and Iuc$^-$ Iut$^-$ derivatives, which lacked a functional aerobactin iron acquisition system but retained catechol production (Table 5), did not grow on the medium. Thus, the aerobactin iron acquisition system contributes significantly to the capacity of strain W3C105 for iron-limited growth. Our results provide the first evidence for a functional aerobactin iron acquisition system in a plant-pathogenic bacterium.

TABLE 5. Characteristics of Iuc$^-$ and Iuc$^-$ Iut$^-$ derivatives of *E. carotovora* W3C105

E. carotovora strain	Phenotype	Aerobactin production[a]	Aerobactin utilization	Iron-limited growth	Generation time (min)	Catechol production[b] (μM)
W3C105	Iuc$^+$ Iut$^+$	+	ND[c]	+	58	3.6
JL11,178	Iuc$^-$ Iut$^+$	−	+	−	64	4.5
JL11,178(pJEL1534)	Iuc$^+$ Iut$^+$	+	+	+	ND	ND
JL11,182	Iuc$^-$ Iut$^-$	−	−	−	64	4.5
JL11,182(pJEL1534)	Iut$^+$ Iut$^+$	+	+	+	ND	ND

[a] Aerobactin production based on hydroxamate production (2) and crossfeeding of *E. coli* LG1522. +, aerobactin production; −, no aerobactin production.
[b] Catechol production assessed by the assay of Rioux et al. (47) and expressed as 2,3-dihydroxybenzoic acid equivalents.
[c] ND, not determined.

The presence of a ferric-aerobactin receptor protein in *E. carotovora* further supports the conclusion that strain W3C105 has a functional aerobactin iron uptake system. The molecular mass of the ferric-aerobactin receptor of *E. carotovora* W3C105 was 80 kDa, in contrast to the 74-kDa receptor protein encoded by pColV-K30 (6, 27–29, 56). Nevertheless, the 80-kDa outer membrane protein of *E. carotovora* was recognized by antiserum raised against the ferric-aerobactin receptor encoded by pColV-K30 and was associated with cloacin DF13 sensitivity, a characteristic of the ferric-aerobactin receptor protein. Although ferric-aerobactin uptake genes of *E. carotovora* conferred both ferric-aerobactin utilization and cloacin DF13 sensitivity on *E. coli*, *E. carotovora* W3C105 itself was not sensitive to cloacin DF13. The tolerance of strain W3C105 to cloacin DF13 may be explained by different conformations or availabilities of the receptor protein in the membrane environments of *E. coli* and *E. carotovora* or detoxification or another resistance response expressed by *E. carotovora* but not by *E. coli*. *E. coli* BN3040 Nal^r grew on an iron-limited medium only if it harbored a plasmid containing both the aerobactin production and uptake genes from either *E. coli* or *E. carotovora* (Fig. 1A), providing further evidence that the genetic determinants of a functional aerobactin iron uptake system are present in *E. carotovora* and were cloned in this study.

Aerobactin-mediated iron uptake is prevalent among the *Enterobacteriaceae*, yet the genetic determinants of aerobactin biosynthesis and uptake vary among these bacteria (41). Aerobactin biosynthesis genes of plasmid or chromosomal origin appear to be highly conserved among *E. coli* and *Shigella* spp. (8, 31, 36, 37, 52); the aerobactin biosynthesis genes of *Aerobacter aerogenes* (58) and *Enterobacter cloacae* (14), however, are distinct from those of *E. coli*. The aerobactin biosynthesis genes of *Enterobacter cloacae* do not hybridize to those of pColV-K30 (14). The aerobactin biosynthesis genes of *E. carotovora* W3C105 hybridized but were not identical to those of *E. coli* (Fig. 2). For example, the aerobactin genes from *E. carotovora* W3C105 were located on an 11-kb *Hin*dIII-*Eco*RI fragment, whereas those of *E. coli* are located on a 7-kb *Hin*dIII-*Eco*RI fragment. The reported restriction maps of the aerobactin genes from *A. aerogenes* (58) and *Enterobacter cloacae* (14) also differ from the map proposed here for *E. carotovora* W3C105 (Fig. 1B). The aerobactin biosynthesis and uptake genes of *E. carotovora* are likely to be of chromosomal origin, since the sole plasmid isolated from *E. carotovora* W3C105 did not hybridize to the aerobactin genes of *E. coli*. The possibility remains, however, that an unidentified plasmid, which was not detected by the standard plasmid extraction procedures used in this study, may encode aerobactin biosynthesis in *E. carotovora* W3C105. The aerobactin system in pColV-K30 is flanked by two IS*1* elements (31, 37, 42). In *E. carotovora*, no IS*1*-like elements were detected by Southern analysis.

In *E. coli*, iron stress induces a coordinated response that is controlled by the ferric uptake regulation (Fur) protein, which acts as a repressor employing iron(II) as a cofactor to bind to the operators of iron-regulated genes (3). At present, we have but a cursory understanding of iron regulation of the siderophore production and uptake systems of *Erwinia* spp. A *trans*-acting factor that influences the iron-regulated production of chrysobactin in *Erwinia chrysanthemi* has been proposed (19), although the relationship of this factor to the Fur protein of *E. coli* is unknown. In *E. carotovora*, production of aerobactin and the outer membrane receptor protein for the ferric aerobactin complex (Fig. 3 and 4) was also regulated by iron. Genes encoding the aerobactin iron acquisition system of *E. carotovora* conferred iron-regulated aerobactin production on *E. coli* DH5α (Table 4). Regulation of the aerobactin genes of *E. carotovora* may be controlled by a protein analogous to the Fur protein, which controls transcription of the aerobactin genes of *E. coli* (3, 11, 17, 53). In *E. coli* BN3040 Nal^r, however, the genes determining the aerobactin iron acquisition system of *E. carotovora* were only slightly regulated by iron (Table 4). Similarly, iron regulation of cloned aerobactin genes from pColV-K30 or *S. flexneri* was only partially retained in strain BN3040 Nal^r (7) and strain BC3 (53), respectively. The presence of the aerobactin genes on a multicopy plasmid is known to interfere with regulation by iron (9, 53). It is unlikely that the poor regulation by iron that was observed here can be explained by high plasmid copy number, however, since the cosmid vector pLAFR3 is generally present in only five to seven copies per cell (21), and the aerobactin genes from pColV-K30 cloned on a plasmid related to pLAFR3 were iron regulated (9).

The ecological significance of siderophore production to plant-pathogenic bacteria is largely unknown (33, 35). Siderophores are produced by every plant-pathogenic organism that has been evaluated (35), yet only chrysobactin (44), produced by *Erwinia chrysanthemi*, has been identified as a virulence factor in plant disease (18). Most of the pathogenic *E. carotovora* strains in this study did not produce aerobactin, suggesting that aerobactin production is not required for the pathogenicity of this bacterium. Nevertheless, other ecological advantages may be associated with a functional aerobactin iron acquisition system. To cause soft-rot diseases, *E. carotovora* must grow and establish high populations intercellularly and on root and tuber surfaces, where it competes with resident microflora for iron and other essential nutrients. *E. carotovora* W3C105 was not as amenable to siderophore-mediated antagonism by fluorescent pseudomonads as was strain W3C37 (63), which does not produce a hydroxamate siderophore (Table 2). It is possible that the aerobactin iron acquisition system of *E. carotovora* W3C105 enhances the ability of this strain to compete with pyoverdine-producing *Pseudomonas* spp. and thus decreases its sensitivity to siderophore-mediated biological control. Knowledge of the role of iron acquisition in the virulence and ecology of target phytopathogens will enhance our current understanding of siderophore-mediated iron competition as a mechanism in biological control. The findings of this study will facilitate future elucidation of the significance of aerobactin production by *E. carotovora* in the virulence, ecology, and control of this pathogen. It is likely that aerobactin has an additional ecological role beyond that as a virulence factor in clinical enteric isolates.

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