Pathogenicity of Mutants of *Erwinia carotovora* subsp. *carotovora* Deficient in Aerobactin and Catecholate Siderophore Production

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ABSTRACT

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Erwinia carotovora subsp. *carotovora* strain W3C105 produces the hydroxamate siderophore aerobactin and a catecholate metabolite, which previously was not known to function in iron acquisition by the phytopathogen. A region containing catechol biosynthesis genes (*cbsEA*$^+$) of strain W3C105 complements EntA$^-$ and EntE$^-$ mutants of *Escherichia coli*, which are deficient in biosynthesis of the catecholate siderophore enterobactin. Four plasmids containing the *cbsEA* region mutagenized with Tn*3*-Spice complemented either the EntA$^-$ or the EntE$^-$ mutant of *E. coli* but not both mutants. Two of the plasmids, in which insertions of Tn*3*-Spice generated transcriptional fusions of the promoterless ice nucleation reporter gene (*inaZ*) to a *cbs* promoter(s), conferred iron-regulated ice nucleation activity on *E. coli*. One of the Tn*3*-Spice insertions was introduced into the genome of W3C105 by marker-exchange mutagenesis to generate a mutant deficient in catechol biosynthesis (Cbs$^-$). Introduction of the insertion into a previously-derived mutant of W3C105 deficient in aerobactin biosynthesis (Iuc$^-$) resulted in a double mutant that produced neither the catechol nor aerobactin (Cbs$^-$ Iuc$^-$). A catechol-producing mutant (Cbs$^+$ Iuc$^-$) grew on a minimal medium containing higher concentrations of the iron-chelator 2,2′-dipyridyl than did a mutant producing neither siderophore (Cbs$^-$ Iuc$^-$), demonstrating that the catechol functions as a siderophore in strain W3C105. Wild-type W3C105 and an aerobactin-producing mutant (Cbs$^-$ Iuc$^+$), however, grew on a minimal medium containing higher concentrations of 2,2′-dipyridyl than did a mutant producing only the catechol (Cbs$^+$ Iuc$^-$), indicating that aerobactin was superior to the catecholate siderophore in competing with 2,2′-dipyridyl for iron. Mutants deficient in the production of either or both siderophores did not differ from the wild-type strain in the capacity to macerate potato tuber tissue or cause aerial stem rot of potato.

Additional keyword: chrysobactin.

Erwinia carotovora subsp. *carotovora* is an economically important bacterial phytopathogen that causes tuber soft rot and aerial stem rot of potato (*Solanum tuberosum* L.) (36,37). After entry into the plant through wounds or natural openings, *E. carotovora* subsp. *carotovora* produces a battery of depolymerizing enzymes that degrade cell walls, resulting in maceration of infected tissues (3). In addition to secretion of enzymes required for virulence, *E. carotovora* subsp. *carotovora* and the related phytopathogen *E. chrysanthemi* produce extracellular siderophores (22,26,38). Siderophores are low molecular weight, iron-chelating compounds that serve as iron transport vehicles (49). Siderophores produced under iron-limited conditions are secreted into the environment, where they specifically chelate the limited amounts of iron(III) that are available (19,48). The ferric-siderophore complex is recovered by the bacteria via a specific outer membrane receptor (34). Iron released from the complex intracellularly becomes available for cellular functions.

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Erwinia spp., like other members of the family Enterobacteriaceae, may produce catecholate siderophores, such as chrysobactin (38), and hydroxamate siderophores, such as aerobactin (22). Catechol production appears to be a ubiquitous phenotype of strains of *E. carotovora* subsp. *carotovora*, whereas aerobactin is produced by a small proportion of strains (22,24). Chrysobactin production is necessary for systemic virulence of *E. chrysanthemi* strain 3937 in *Saintpaulia ionantha* (11,12), but the function of catechol(s) produced by *E. carotovora* subsp. *carotovora* in iron acquisition and virulence of the phytopathogen has not been demonstrated. Our studies focus on *E. carotovora* subsp. *carotovora* strain W3C105, which produces aerobactin (22) and a catechol (5). Aerobactin biosynthesis (*iuc*) and ferric-aerobactin uptake (*iut*) genes have been cloned from W3C105, and mutants deficient in aerobactin biosynthesis (Iuc$^-$) and uptake (Iut$^-$) have been derived. These mutants exhibit a reduced capacity to grow on iron-limited media (22). Catechol biosynthesis genes also have been identified from a genomic library of W3C105 by functional complementation of mutants of *Escherichia coli* that are deficient in biosynthesis of the tricatecholate siderophore enterobactin (Ent$^-$) (5). In this study, we investigated the relative roles of catechol and aerobactin production in iron-limited growth and pathogenicity of W3C105. Portions of this work were reported in an abstract (4).

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in the study are listed in Table 1. Rifampicin-resistant derivatives of these strains were described previously (5). Strains of *E. coli* and *E. chrysanthemi* were cultured on Luria-Bertani medium (LB) (17) at 37°C, and *E. carotovora* subsp. *carotovora* was cultured on LB medium at 26°C. Catechol and aerobactin production was determined from cultures grown in Tris minimal salts medium (TMS) supplemented with tryptophan (0.003% wt/vol), thiamine (0.0002% wt/vol), and casamino acids (0.3% wt/vol), from which contaminating iron was removed by extraction with 8-hydroxyquinoline, as described previously (23). The iron availability of TMS medium was enhanced by adding $FeCl_3$ (100 or 0.1 μM final concentrations) or decreased by adding the chelator 2,2′-dipyridyl (105, 120, 135, or 150 μM final concentrations). $FeCl_3$ and 2,2′-dipyridyl were added as filter-sterilized solutions after the medium was autoclaved. Antibiotics were used at the following concentrations: ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, nalidixic acid at 100 μg/ml, rifampicin at 100 μg/ml, spectinomycin at 50 μg/ml, streptomycin at 100 μg/ml, and tetracycline at 20 μg/ml. All chemicals were purchased through Sigma Chemical Co., St. Louis, unless otherwise specified.

Molecular genetics and recombinant DNA techniques. Transformation of *E. coli* was performed according to the procedure described for *E. coli* DH5α by Gibco-BRL Life Technologies, Gaithersburg, MD. Plasmids were mobilized into *E. carotovora* subsp. *carotovora* or rifampicin-resistant strains of *E. coli* from *E. coli* S17-1 (45) or in triparental matings using the helper plasmid pRK2013 (15). Transconjugants of *E. carotovora* subsp. *carotovora* were selected on crystal violet pectate agar (CVP) (8) containing appropriate antibiotics.

Plasmids were isolated from *E. coli* and *E. carotovora* subsp. *carotovora* by an alkaline-lysis extraction procedure and purified by cesium chloride density-gradient centrifugation for Southern hybridization and cloning experiments (41). Genomic DNA was isolated by a cetyltrimethylammoniumbromide method (2). Restriction endonucleases were used according to recommendations of the supplier (Gibco-BRL). Restriction fragments were separated by gel electrophoresis in 0.7% (wt/vol) agarose gels (SeaKem LE, FMC BioProducts, Rockland, ME) in Tris-phosphate-EDTA buffer (41). For Southern blots, DNA was transferred from agarose gels to nylon membranes (Nytran, Scheicher & Schuell, Keene, NH) by standard methods (41). Fragments used as probes were purified from agarose gels (SeaKem GTG, FMC) by adsorption and elution from NA-45 DEAE membranes (Scheicher & Schuell). Probes were prepared by nick translation of the fragments with ^{32}P-dCTP (New England Nuclear, Boston) and were used at a concentration of 25 ng/ml.

Tn*3*-Spice mutagenesis of cloned catechol-biosynthesis genes (*cbs*). The transposon Tn*3*-Spice contains a promoterless gene conferring ice nucleation activity (*inaZ*) and can be used to generate transcriptional fusions with genes into which it is inserted. Tn*3*-Spice insertions into pJEL1602 were generated by published methods (27). Individual pJEL1602::Tn*3*-Spice plasmids were mobilized into rifampicin-resistant derivatives of *E. coli* strains AN93 and AN193 to evaluate complementation of the *entE* and *entA* mutations of *E. coli*, respectively. From four pJEL1602::Tn*3*-Spice plasmids, the *Hin*dIII fragment containing the β-lactamase gene in Tn*3*-Spice and a contiguous region of pJEL1602 was subcloned to facilitate restriction mapping of the insertion sites. Ice nucleation activity conferred by pJEL1602::Tn*3*-Spice plasmids was measured at –5°C by a droplet-freezing technique (28) from cultures grown at 26°C for 48 h in 5 ml of TMS broth amended with $FeCl_3$ or 2,2′-dipyridyl. Ice nucleation activity was determined from three replicate cultures, and the experiment was repeated.

Derivation of mutants of *E. carotovora* subsp. *carotovora* deficient in catecholate siderophore production (Cbs^-). The Tn*3*-Spice insertion of pJEL1752 (Fig. 1) was introduced into the genome of strain W3C105 and aerobactin-deficient derivatives JL11178 (Iuc^-) and JL11182 (Iuc^- Iut^-) by marker-exchange mutagenesis as described previously (22). W3C105 containing pJEL1752 was grown at 26°C, with shaking in LB broth (200 ml in a 1-liter flask). After 12 h, 100 μl of the culture was transferred to fresh LB broth (200 ml in a 1-liter flask). After six successive transfers, spectinomycin-resistant, tetracycline-sensitive colonies were selected and compared to parental strains for pectolytic activity on CVP agar, growth on TMS, and serological identity to strain W3C105 (9). Antiserum specific to serogroup 39 of *E. carotovora* subsp. *carotovora* was provided by M. Powelson (Oregon State University, Corvallis). The site of the Tn*3*-Spice insertion was confirmed from Southern blots of genomic DNA of marker-exchange mutants probed with an 8.8-kb *Kpn*I fragment of pJEL1752 containing 1.8 kb of *inaZ*. Generation times of mutants and parental strains were determined by the change in optical density at 600 nm of cultures grown with shaking in LB broth at 26°C.

Detection of siderophore production. Siderophore production by bacterial strains was detected by observation of orange halos surrounding bacterial colonies grown on an agar medium containing chrome azurol S (CAS) (43). CAS agar is considered a universal siderophore-detection medium (43) because siderophores are detected on the basis of their capacities to remove iron from the medium rather than by the presence of a specific chemical group involved in iron chelation. Catechol and hydroxamate concentrations were quantified from supernatants of bacterial cul-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Escherichia coli:		
DH5α	F^-, *end*A1, *hsd*17 (r_k^-, m_k^+), *supp*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *φ80dlac*ZΔ, m15λ, Ent^+	Gibco-BRL
S17-1	Tra^+, Ent^+, Sm^r	45
AN194	F^-, *ton*A23, *pro*C14, *leu*16, *trp*E38, *thi*-1, Ent^+, Sm^r	25
AN93	as AN194 but *ent*E405	43
AN193	as AN194 but *ent*A403	47
Erwinia carotovora subsp. *carotovora*:		
W3C105	Field isolate, serogroup 39, Cbs^+, Iuc^+, Iut^+	50
JL11178	W3C105 Δ*iuc*, Cbs^+, Iuc^-, Iut^+	22
JL11182	W3C105 Δ (*iuc-iut*), Cbs^+, Iuc^-, Iut^-	22
LA202	W3C105 *cbs*::Tn*3*-Spice, Cbs^-, Iuc^+, Iut^+	This study
LA206	W3C105 Δ*iuc cbs*::Tn*3*-Spice, Cbs^-, Iuc^-, Iut^+	This study
LA207	W3C105 Δ (*iuc-iut*) *cbs*::Tn*3*-Spice, Cbs^-, Iuc^-, Iut^-	This study
SR319	Isolated from soil in Wisconsin, serogroup 29, Cbs^+	A. Kelman
SCRI-193	Isolated from soil in Scotland, Cbs^+	21
Plasmid:		
pRK2013	Tra^+, Km^r	15
pLAFR3	*inc*P1 replicon, *cos*, Tc^r	46
pJEL1534	iuc^+ iut^+ region of *E. c.* subsp. *carotovora* W3C105 cloned in pLAFR3	22
pJEL1602	*cbs(DC)EA*$^+$ region of *E. c.* subsp. *carotovora* W3C105 cloned in pLAFR3	5
pJEL1742	pJEL1602::Tn*3*-Spice #42, $cbsA^-$, $cbsE^+$	This study
pJEL1751	pJEL1602::Tn*3*-Spice #51, $cbsA^-$, $cbsE^+$	This study
pJEL1752	pJEL1602::Tn*3*-Spice #52, $cbsE^-$, $cbsA^+$	This study
pJEL1755	pJEL1602::Tn*3*-Spice #55, $cbsA^-$, $cbsE^+$	This study
pJEL1868	5.6-kb *Kpn*I fragment from pJEL1602 cloned in pUC19, $cbsEA^+$	5

[a] Abbreviations: Ent^+ and Ent^-, enterobactin producer or nonproducer, respectively; Iuc^+ and Iuc^-, aerobactin producer or nonproducer, respectively; Iut^+ and Iut^-, possesses or lacks, respectively, the outer membrane receptor for ferric aerobactin; Cbs^+ and Cbs^-, catechol producer or nonproducer, respectively; and Tra^+, conjugal transfer. Antibiotic resistances: Km^r, kanamycin; Sm^r, streptomycin; Tc^r, tetracycline.

tures grown for 24 to 48 h in TMS as described by Arnow (1) or Rioux et al. (40) and Csáky (7), respectively.

Cross-feeding of siderophore production mutants of *E. coli* and *E. carotovora* subsp. *carotovora*. Catecholate siderophore production was detected by a cross-feeding bioassay that relies on the inability of indicator strains, which have mutations in siderophore biosynthetic genes, to grow on an iron-limiting medium unless a utilizable form of iron is provided from an exogenous source (35). The cross-feeding bioassay also was used to evaluate siderophore utilization by mutants of *E. carotovora* subsp. *carotovora* used as indicator strains. Molten TMS agar containing 140 μM 2,2′-dipyridyl was inoculated with an aqueous suspension of an indicator strain to a final cell density of 10^5 CFU/ml. Strains to be tested for catecholate siderophore production were grown overnight with shaking in TMS broth, and 10 μl of the culture was spotted on the surface of solidified, inoculated TMS medium. After 24 to 48 h of incubation at 26°C, plates were observed for growth of the indicator strain surrounding growth of the test strain. *E. coli* AN194, which produces enterobactin, was included as a test strain to provide a positive control. Two replicate plates were evaluated, and the experiment was repeated. LSD ($P = 0.05$) was used for analysis of mean separation.

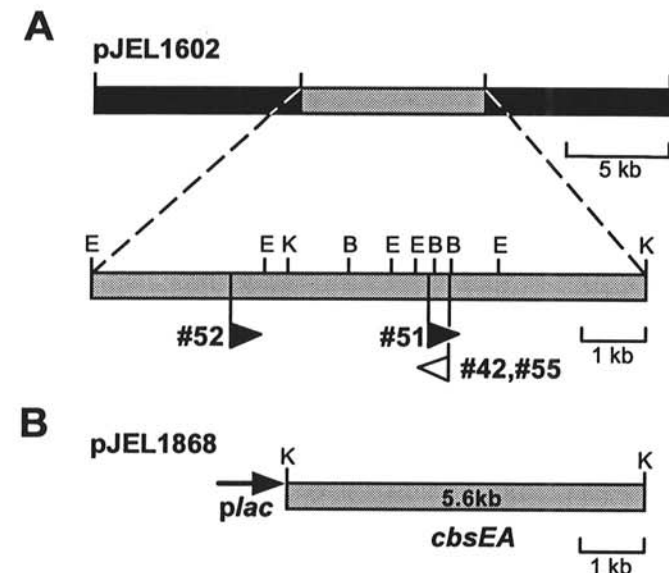

Fig. 1. A, Location of Tn*3*-Spice insertions in plasmid pJEL1602. The region of pJEL1602 containing insertions is enlarged. Flags indicate the location of insertions in pJEL1602::Tn*3*-Spice plasmids pJEL1752 (#52), pJEL1751 (#51), pJEL1742 (#42), and pJEL1755 (#55). Locations of the Tn*3*-Spice insertions of pJEL1742 and pJEL1755 were not distinguishable from one another by restriction mapping. pJEL1752 and pJEL1751 conferred iron-regulated ice nucleation activity on *Escherichia coli*, as indicated by filled flags. pJEL1742 and pJEL1755 conferred only low, iron-constitutive levels of ice nucleation activity on *E. coli*, as indicated by an open flag. Restriction endonuclease sites are abbreviated: B = *Bam*HI; E = *Eco*RI; K = *Kpn*I. **B,** Plasmid pJEL1868, which is composed of the 5.6-kb *Kpn*I fragment from pJEL1602 that complements the *entE* and *entA* mutations of *E. coli* cloned in pUC19 (5). *plac* denotes the location of the *lac* promoter present in the multiple cloning site of pUC19.

TABLE 2. Characterization of plasmid pJEL1602 and derivatives containing insertions of Tn*3*-Spice

	Complementation of Ent⁻ mutants of *Escherichia coli*[a]		Ice nucleation activity[b] $\log_{10}$(ice nuclei/cell)	
Plasmid	AN93 (EntE⁻)	AN193 (EntA⁻)	0.1 μM $FeCl_3$	100.0 μM $FeCl_3$
pJEL1602	+	+	nd	nd
pJEL1742	+	–	–8.0	–7.6
pJEL1751	+	–	–1.1	–4.7
pJEL1752	–	+	–0.6	–3.6
pJEL1755	+	–	–5.7	–6.0

[a] + = catechol siderophore production was detected by colorimetric assays (1, 40), by a bioassay evaluating cross-feeding of *E. coli* AN93, and by observation of an orange halo surrounding the bacterial colony on chrome azurol S agar (43); – = no catechol siderophore production detected by these methods.

[b] Ice nucleation activity at –5°C. Values are mean activities expressed by three replicate cultures of *E. coli* DH5α harboring specified plasmids. Cultures were grown in Luria-Bertani medium for 48 h at 26°C. nd = ice nucleation activity was not detected.

Tuber soft rot assay. Potato tubers (cv. Russet Burbank) were surface-disinfested by soaking tubers for 20 min in 0.05% sodium hypochlorite solution and rinsing thoroughly with sterile deionized H_2O prior to inoculation. Inoculum was a bacterial suspension adjusted to an optical density of 0.1 at 600 nm in 0.1 M $MgSO_4$ (approximately 10^8 CFU/ml) and a 1:1,000 dilution of the suspension (approximately 10^5 CFU/ml). A pipette tip containing 100 μl of a bacterial suspension was used to stab a potato tuber as described by De Boer and Kelman (10). The pipette tip was left in place while tubers were incubated in a moist chamber at 22°C for 4 days. Disease was evaluated by weighing the rotted tissue surrounding the inoculation site. Treatments were replicated 10 times, and the experiment was repeated. Experiments were designed and analyzed as completely randomized experiments. Data were analyzed by the general linear models procedure of Statistical Analysis Systems (SAS Institute, Cary, NC). Tukey's range test ($P = 0.05$) was used for analysis of mean separation.

Aerial stem rot assay. Inoculum was a suspension of bacterial cells grown on LB agar that was adjusted to an optical density of 0.1 at 600 nm in 0.1 M phosphate buffer (pH 7.0) and diluted 1:20 in the phosphate buffer. A bacterial suspension (125 μl; approximately 5×10^6 CFU/ml) or sterile phosphate buffer was placed on a sterile disk (50 mm diameter) of absorbent paper (No. 740-E, Schleicher & Schuell). The disk was placed directly on a wound in the stem of a potato plant (cv. Norcotah) and held in place with polypropylene packing tape. The wound was inflicted by removing the leaf and petiole located 5 to 6 leaves from the plant apex and stabbing the scar with a sterile needle. Sixteen replicate plants were inoculated with each bacterial strain. Sixteen control plants were inoculated with sterile phosphate buffer.

After inoculation, plants were placed randomly in plastic trays lined with moist paper towels. Plants in trays were covered loosely with a plastic bag and placed in a greenhouse (22°C). Plastic bags were removed 48 h after inoculation, and stem rot symptoms were evaluated visually at 12-h intervals for the next 4 days. Symptoms were rated on a 1 to 5 scale according to the following criteria: 1 = no visible stem lesion; 2 = visible stem lesion (i.e., lesions extending >1 cm beyond wound site were easily differentiated from wound scars), no visible wilt symptoms; 3 = visible stem lesion, wilt in less than half of leaves above wound site; 4 = visible stem lesion, wilt in more than half of leaves above wound site; 5 = visible stem lesion, plant above wound site was without turgor. We compared disease severity ratings of treatments at a given time point using Wilcoxson scores ($P = 0.05$) calculated by the NPAR1way procedure of SAS. We also calculated the area under the disease progress curve (AUDPC) (44) from repeated aerial stem rot ratings of each replicate plant. Statistical differences ($P = 0.05$) between mean AUDPC values of various treatments were determined by the general linear models procedure of SAS. The experiment was done three times. Results from each of the experiments were similar, and a representative experiment is presented.

RESULTS

Analysis of Tn*3*-Spice insertions in cloned catecholate biosynthesis genes of *E. carotovora* subsp. *carotovora*. Insertions of Tn*3*-Spice into certain regions of pJEL1602 inactivated functions required for complementation of enterobactin biosynthesis

mutants of *E. coli*. Plasmids pJEL1742, pJEL1751, and pJEL1755, which had insertions of Tn*3*-Spice within 1 kb of one another (Fig. 1A), did not complement the *entA* mutation of *E. coli* (Table 2). The insertions in these plasmids were located within a 5.6-kb *Kpn*I fragment required for complementation of *entA* and *entE* mutations of *E. coli* (Fig. 1B). Plasmid pJEL1752, which had a Tn*3*-Spice insertion located outside of the 5.6-kb *Kpn*I fragment, did not complement the *entE* mutation of *E. coli*. Plasmids pJEL1751 and pJEL1752 conferred iron-regulated ice nucleation activity on *E. coli* DH5α (Table 2). In contrast, the low levels of ice nucleation activity conferred by plasmids pJEL1742 and pJEL1755 were not influenced by the level of available iron. None of the four plasmids conferred detectable levels of ice nucleation activity on *E. carotovora* subsp. *carotovora* strain W3C105.

Derivation of Cbs⁻ mutants of *E. carotovora* subsp. *carotovora*. Cbs⁻ mutants were derived by introducing the Tn*3*-Spice insertion of pJEL1752 into the genome of W3C105 and its Iuc⁻ derivatives. Southern hybridizations confirmed that each Cbs⁻ mutant had a single genomic insertion of Tn*3*-Spice corresponding to the site in pJEL1752 (data not shown). Cbs⁻ mutants did not produce catechol, were prototrophic, produced pits on CVP medium, and had growth rates in LB broth similar to W3C105. Cbs⁻ mutants did not express detectable ice nucleation activity. Strains deficient in both catechol and aerobactin production (Cbs⁻ Iuc⁻; i.e., LA206 and LA207) did not produce halos on CAS agar, indicating a lack of siderophore production on that medium (Table 3). Catechol production was restored to the Cbs⁻ mutants by introducing pJEL1602, which contains cloned catechol-biosynthesis genes from W3C105. Aerobactin production was restored to Iuc⁻ mutants by introducing pJEL1534, which contains the cloned aerobactin-biosynthesis and uptake genes from W3C105.

Growth of *E. carotovora* subsp. *carotovora* on iron-limiting media. Strain W3C105 and Cbs⁻ derivatives that produced aerobactin grew on an iron-limited medium (TMS amended with 150 μM 2,2′-dipyridyl) (Table 3). Mutants lacking a functional aerobactin iron-acquisition system (JL11178, JL11182, LA206, and LA207) did not grow on TMS containing 150 μM 2,2′-dipyridyl irrespective of catechol production. Plasmid pJEL1534, which contains genes for aerobactin biosynthesis and uptake, restored the capacity of these strains to grow on TMS containing 150 μM 2,2′-dipyridyl. Strains deficient in both catechol and aerobactin production (LA206 and LA207) grew on TMS containing concentrations of ≤120 μM 2,2′-dipyridyl but did not grow on TMS containing 135 or 150 μM 2,2′-dipyridyl. Introduction of pJEL1602, which contains catechol biosynthesis genes, restored the capacity of these mutants to grow on TMS containing 135 μM 2,2′-dipyridyl, demonstrating that the catechol functions as a siderophore in W3C105. Because the wild-type strain of W3C105 and an aerobactin-producing mutant (Cbs⁻ Iuc⁺) grew on TMS containing higher concentrations of 2,2′-dipyridyl than did a mutant producing only the catechol (Cbs⁺ Iuc⁻), aerobactin was superior to the catecholate siderophore in competing with 2,2′-dipyridyl for iron.

Cross-feeding of siderophore production mutants of *E. coli* and *E. carotovora* subsp. *carotovora*. *E. carotovora* subsp. *carotovora* strain W3C105 and Cbs⁺ derivatives cross-fed the EntA⁻ mutant *E. coli* AN193 and strain LA207 (Cbs⁻ Iuc⁻ Iut⁻) of *E. carotovora* subsp. *carotovora* (Table 4). Strain LA202 (Cbs⁻ Iuc⁺ Iut⁺), which did not produce catechol, did not cross-feed *E. coli* AN193 or *E. carotovora* subsp. *carotovora* LA207. W3C105 and derivatives that produced either aerobactin or a catecholate siderophore cross-fed strain LA206 (Cbs⁻ Iuc⁻ Iut⁺). The aerobactin-producing strains W3C105 and LA202 produced larger zones of cross-feeding of LA206 than did near-isogenic test strains that produced only catechol. Two heterologous strains of *E. carotovora* subsp. *carotovora* (SCRI193 and SR319) and the enterobactin-producing strain AN194 of *E. coli* cross-fed both LA206 and LA207, indicating that utilization of siderophores produced by these heterologous strains did not depend on the ferric-aerobactin receptor protein. The zone sizes surrounding these heterologous strains were larger than those surrounding W3C105 or derivative test strains. Reasons for the large size of these cross-feeding zones were not evaluated here but include the possibilities that a siderophore(s) produced by heterologous strains was highly effective for iron acquisition of LA206 and LA207, was exceptionally stable, diffused readily into the medium, or was produced in large quantities.

Tuber soft rot and aerial stem rot caused by mutants of *E. carotovora* subsp. *carotovora* deficient in siderophore production. Strain W3C105 and derivatives varying in siderophore production did not differ significantly in virulence, assessed by maceration of potato tuber tissue. The weight of macerated tissue

TABLE 3. Iron-limited growth of *Erwinia carotovora* subsp. *carotovora* strain W3C105 and derivatives deficient in siderophore biosynthesis

Strain (Plasmid)[a]	Phenotype	CAS reaction[b]	Growth on 2,2′-dipyridyl[c]		
			120 μM	135 μM	150 μM
W3C105	Cbs⁺ Iuc⁺ Iut⁺	+	+	+	+
JL11178	Cbs⁺ Iuc⁻ Iut⁺	+	+	+	–
JL11178 (pJEL1534)	Cbs⁺ Iuc⁺ Iut⁺	+	nt	nt	+
JL11182	Cbs⁺ Iuc⁻ Iut⁻	+	+	+	–
JL11182 (pJEL1534)	Cbs⁺ Iuc⁺ Iut⁺	+	+	+	+
LA202	Cbs⁻ Iuc⁺ Iut⁺	+	nt	+	+
LA206	Cbs⁻ Iuc⁻ Iut⁺	–	+w	–	–
LA206 (pJEL1534)	Cbs⁻ Iuc⁺ Iut⁺	+	nt	nt	+
LA206 (pJEL1602)	Cbs⁺ Iuc⁻ Iut⁺	+	+	+	–
LA207	Cbs⁻ Iuc⁻ Iut⁻	–	+w	–	–
LA207 (pJEL1534)	Cbs⁻ Iuc⁺ Iut⁺	+	+	+	+
LA207 (pJEL1602)	Cbs⁺ Iuc⁻ Iut⁻	+	+	+	–

[a] pJEL1534 contains cloned genes for aerobactin biosynthesis (Iuc⁺) and the ferric-aerobactin receptor (Iut⁺); pJEL1602 contains cloned genes for catechol biosynthesis (Cbs⁺).

[b] + = an orange halo surrounded bacterial colonies grown on chrome azurol S (CAS) agar, indicating siderophore production; – = no orange halo was observed.

[c] Growth on Tris minimal salts medium (TMS) containing 2,2′-dipyridyl was recorded after 3 and 5 days: + = growth; +w = weak growth; – = no growth; nt = not tested. All strains grew on TMS containing 0 or 105 μM 2,2′-dipyridyl. Each treatment was replicated three times, and the experiment was repeated.

TABLE 4. Cross-feeding of siderophore production mutants of *Escherichia coli* and *Erwinia carotovora* subsp. *carotovora*[a]

Test strain	Phenotype	Cross-feeding of *E. coli* AN193 (EntA⁻)	Cross-feeding of *E. c.* subsp. *carotovora* (radius [mm] of zone)[b]	
			LA206	LA207
E. c. subsp. *carotovora*:				
W3C105	Cbs⁺ Iuc⁺ Iut⁺	+	16	3
JL11178	Cbs⁺ Iuc⁻ Iut⁺	+	5	2
JL11182	Cbs⁺ Iuc⁻ Iut⁻	+	3	2
LA202	Cbs⁻ Iuc⁺ Iut⁺	–	15	0
SCRI193	Cbs⁺	+	10	10
SR319	Cbs⁺	+	16	13
E. coli:				
AN194	Ent⁺	+	10	9

[a] Growth of an indicator strain surrounding a test strain colony was observed 24 to 48 h after inoculation of Tris minimal salts medium containing 140 μM 2,2′-dipyridyl. Each treatment was replicated two times, and the experiment was repeated.

[b] LA206: (Cbs⁻ Iuc⁻ Iut⁺); LA207: (Cbs⁻ Iuc⁻ Iut⁻); LSD (P = 0.05) = 3.

(1.1 to 1.7 g per inoculation site) did not differ significantly among potatoes inoculated with 10^7 CFU of W3C105, JL11178, JL11182, LA202, LA206, or LA207 per wound. Similarly, the weight of macerated tissue (0.3 to 0.7 g per inoculation site) did not differ significantly among potatoes inoculated with 2×10^3 CFU of the strains per wound.

Derivatives of W3C105 deficient in the production of aerobactin, a catechol siderophore, or both siderophores retained the capacity to cause aerial stem rot of potato (Fig. 2). Mean disease severity ratings did not differ significantly among bacterial treatments at any point in time at which aerial stem rot symptoms were evaluated. Calculated AUDPCs also did not differ significantly among treatments, indicating that the virulence of near-isogenic strains differing in siderophore production did not differ significantly.

DISCUSSION

A catechol produced by *E. carotovora*. subsp. *carotovora* W3C105 functioned in iron acquisition by the phytopathogen, as determined by several lines of evidence. (i) Catechol production enabled an Iuc⁻ mutant of W3C105 to grow on an iron-limited medium. (ii) Catechol production was detectable on a universal siderophore-detection medium (CAS). (iii) An exogenous source of the catechol enabled Cbs⁻ Iuc⁻ mutants of W3C105 to grow on an iron-limited medium in cross-feeding experiments. Nevertheless, aerobactin was functionally superior to the catecholate siderophore in iron acquisition by W3C105: Iuc⁻ mutants could not grow on an extremely iron-deplete medium (i.e., TMS containing 150 μM 2,2′-dipyridyl) regardless of catechol production. This result would be surprising if the catechol produced by strain W3C105 was the tricatecholate siderophore enterobactin. Enterobactin has a high affinity for iron (pFe = $10^{35.5}$ at pH 7.4) in comparison to aerobactin (pFe = $10^{23.3}$ at pH 7.4) (32), so its functional superiority in sequestering iron would be expected. Although the structure of the catecholate siderophore produced by *E. carotovora* subsp. *carotovora* W3C105 has not been reported, many of its chemical properties are similar to those of chrysobactin (H. Barnes and C. A. Ishimaru, *personal communication*), the monocatecholate siderophore produced by *E. chrysanthemi* (38). Chrysobactin functions as a bidentate ligand (39) and, therefore, is expected to be a far less efficient chelator than enterobactin (20). In addition to the affinity of a siderophore for iron(III), its concentration and the chemical stability of its ferric complex also have a profound effect on its efficiency in providing iron to a bacterium (29). Therefore, a number of factors may have contributed to the functional superiority of aerobactin over the catecholate siderophore produced by W3C105.

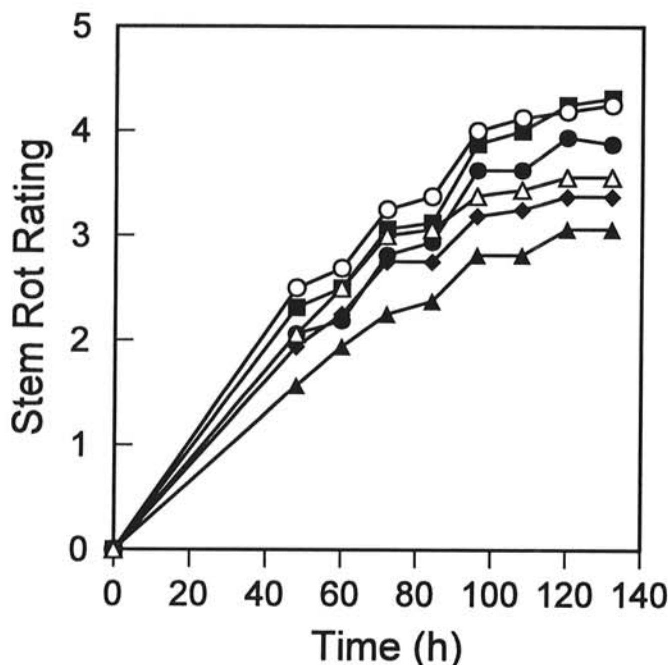

Fig. 2. Aerial stem rot of potato caused by *Erwinia carotovora* subsp. *carotovora* strain W3C105 (■) and derivatives deficient in siderophore production: JL11178 (●), JL11182 (○), LA202 (◆), LA206 (▲), and LA207 (△). At time 0, wounds on stems were inoculated with bacterial cell suspensions (approximately 6×10^5 CFU per wound) in 0.1 M phosphate buffer (pH 7.0). Aerial stem rot symptoms were rated at the times indicated according to a 1 to 5 scale, with 1 being least severe (no visible stem rot lesion) and 5 being most severe (visible stem rot lesion and plant above wound site completely wilted). No stem lesions or wilting were observed on control plants inoculated only with buffer.

In *E. carotovora* subsp. *carotovora* strain W3C105, ice nucleation activity expressed from the introduced *inaZ* fusions, from an *iceC* gene of *Pseudomonas syringae*, or from a promoterless *inaZ* transcribed from a *lac* promoter (C. T. Bull, M. J. Boehm, and J. E. Loper, *unpublished*), was low or not detected. Poor expression of ice nucleation activity by W3C105 was unexpected, because many gram-negative bacteria (27), including other strains of *E. carotovora* subsp. *carotovora* (M. J. Boehm and J. E. Loper, *unpublished*), express ice nucleation activity efficiently. The hydrophobic environment of the bacterial outer membrane is critical to the proper assembly and structural organization of the InaZ protein complex. A membrane that does not support the formation of this complex does not function as an effective ice nucleus (18). We chose the *inaZ* reporter because ice nucleation activity of bacteria in culture or in natural substrates, such as potato tubers, can be conveniently quantified, providing an assessment of gene expression under diverse conditions (27,30). Unfortunately, the capacity of W3C105 to produce functional ice nuclei was not adequate for the evaluation of transcriptional activity using the *inaZ* reporter. Because *E. coli* efficiently expresses ice nuclei from an introduced, transcribed *inaZ* gene (27), transcriptional activity of *cbs* promoter(s) was assessed in that bacterium.

Four Tn*3*-Spice insertions inactivated either *cbsA* or *cbsE* genes, which complement mutations of *E. coli* in the enterobactin biosynthesis genes *entA* and *entE*, respectively. Three of the Tn*3*-Spice insertions (#42, 51, and 55) (Fig. 1) were located within a 5.6-kb *Kpn*I fragment that, if cloned downstream of a *lac* promoter, was sufficient for complementation of the *entA* and *entE* mutations (5). Tn*3*-Spice insertion #52, which inactivated *cbsE*, was located outside this fragment. It is plausible that Tn*3*-Spice insertion #52 had a polar effect on the *cbsE* gene. If so, the *cbsE* and *cbsA* genes must be transcribed from two different promoters, because insertion #52 did not inactivate *cbsA*. Because none of the four Tn*3*-Spice insertions inactivated both *cbsE* and *cbsA* genes, it is likely that these genes are not in a common operon but are transcribed from two distinct promoters in W3C105. Tn*3*-Spice insertions #51 and 52, which inactivated *cbsA* and *cbsE*, respectively, conferred iron-regulated ice nucleation activity on *E. coli*, presumably because the insertions were located downstream of a promoter(s) of the *cbs* operon of *E. carotovora* subsp. *carotovora*. Thus, iron regulation of catecholate siderophore production occurs, at least in part, at the level of *cbs* gene transcription, as has been observed in *E. chrysanthemi* (14,31). Tn*3*-Spice insertions #42 and 55, which were in the opposite orientation to insertions #51 and 52, conferred low levels of iron-constitutive ice nucleation activity on *E. coli*, which may represent background levels of transcription. Taken together, these data indicate that *cbsE* and *cbsA* are members of a gene cluster that is transcribed from left to right as viewed in Figure 1. As such, the gene cluster is similar to the *entCEBA* operon of *E. coli* (6) and the *fct-cbsCEBA* operon of *E. chrysanthemi* (16,31) in the order of *cbsE* and *cbsA* relative to the direction of their transcription.

Production of aerobactin or the catecholate siderophore was not essential for *E. carotovora* subsp. *carotovora* strain W3C105 to

macerate potato tuber tissue or to cause aerial stem rot of potato. Although it is possible that *E. carotovora* subsp. *carotovora* produces an additional siderophore in planta that could have compensated for the loss of known siderophore production, aerobactin and the catechol were the only siderophores produced by W3C105 on a culture medium (i.e., CAS agar) (43) designed to detect all classes of microbial siderophores.

Levels of iron that are available to *E. carotovora* subsp. *carotovora* in potato tubers or stems are not known, but iron availability in intercellular fluids of leaves of *S. ionantha* is sufficiently low to be conducive to chrysobactin production and transcription of the iron-regulated promoter of the *fct-cbs* operon by *E. chrysanthemi* (31,33). Iron deprivation restricts the growth of *E. chrysanthemi* but enhances pectate lyase activity and transcription of genes encoding pectate lyases in culture (42). If mutants lacking a functional chrysobactin iron-acquisition system have depleted levels of iron available for cellular functions, then their capacity to macerate tissue should reflect a balance between restricted growth and enhanced pectate lyase production. *E. chrysanthemi* does not require a functional chrysobactin iron-acquisition system to macerate leaf tissue of *S. ionantha*. In contrast, a functional chrysobactin iron-acquisition system is required for systemic virulence of *E. chrysanthemi* (12) and contributes to the growth of the phytopathogen in intercellular spaces (13).

In this study, we found no evidence that production of aerobactin or a catechol siderophores was a virulence factor of *E. carotovora* subsp. *carotovora* W3C105. Severity of tuber soft rot or aerial stem rot caused by mutants of W3C105 that produced neither siderophore was similar to that caused by the parental strain, which produced both siderophores. We recognize, however, that virulence assays, even when carefully designed and analyzed, can only approximate, not duplicate, the conditions under which disease occurs in nature. Therefore, we cannot exclude the possibility that high-affinity iron acquisition systems play a role in the virulence or ecology of *E. carotovora* subsp. *carotovora* in some natural habitats.

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