Characterization of Siderophore Production by the Biological Control Agent *Enterobacter cloacae*

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Received 21 October 1993. Revision received 10 March 1994. Accepted 25 March 1994.

***Enterobacter cloacae* EcCT-501, which suppresses Pythium damping-off of cucumber and other plant hosts, produces the hydroxamate siderophore aerobactin and a catechol siderophore tentatively identified as enterobactin. Cloned fragments of genomic DNA of *E. cloacae* EcCT-501 complemented the enterobactin (*ent*) biosynthesis mutations *entA, entB, entC, entE,* and *entF* of *Escherichia coli.* By complementation of *ent* mutations of *E. coli,* we localized the *ent(CEB)A* region to a 4.5-kb *Hin*dIII-*Xho*I fragment of the *E. cloacae* genome and the *entA* gene to a 1.8-kb *Eco*RI-*Xho*I fragment within the *ent(CEB)A* region. We deleted a 0.8-kb *Eco*RV fragment internal to the *entA* genomic region of *E. cloacae* EcCT-501 to construct mutants deficient in enterobactin production (Ent$^-$). Introduction of this deletion into a derivative of EcCT-501 that was deficient in aerobactin production (Iuc$^-$) resulted in a mutant deficient in the production of both enterobactin and aerobactin (Ent$^-$ Iuc$^-$). Strain EcCT-501 and the Ent$^-$ Iuc$^+$ and Ent$^+$ Iuc$^-$ derivatives grew on an iron-limited medium. The Ent$^-$ Iuc$^-$ mutant, however, failed to grow on the iron-limited medium, indicating that both aerobactin and enterobactin function in iron acquisition by *E. cloacae.* Aerobactin and enterobactin production by *E. cloacae* did not contribute to its activity in biological control of Pythium damping-off of cotton or cucumber.**

Enterobacter cloacae is a biological control agent of *Pythium ultimum,* a prevalent fungal phytopathogen that causes damping-off of many crop plants (Hadar *et al.* 1983; Howell *et al.* 1988; Nelson *et al.* 1986; Nelson 1988). Although mechanisms by which *E. cloacae* protects seeds from infection by *P. ultimum* are unknown, four hypotheses to explain this suppression have been proposed. 1) The bacterium attaches to hyphae of the fungus, forming sheaths of bacterial cells that inhibit hyphal development (Nelson *et al.* 1986). 2) The bacterium produces ammonia, which inhibits fungal growth (Howell *et al.* 1988). 3) The bacterium utilizes plant exudates required for sporangial germination of *P. ultimum,* thus reducing root infection (Nelson and Maloney 1992). 4) The bacterium produces siderophores that inhibit the growth of *P. ultimum* through iron competition (Loper *et al.* 1993).

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 (Neilands 1981). Most siderophores contain hydroxamate or catechol groups that form all or part of the iron chelation center of the molecules (Neilands and Nakamura 1991). Siderophores produced by certain biocontrol agents are thought to deplete available iron in a pathogen's microenvironment by sequestering ferric ions as ferric-siderophore complexes, which the pathogen cannot utilize (Loper and Buyer 1991; O'Sullivan and O'Gara 1992; Schippers *et al.* 1987). *P. ultimum* is sensitive to siderophore-mediated iron competition from *Pseudomonas* spp. (Becker and Cook 1988; Loper 1988) and may also be sensitive to iron deprivation imposed by siderophores produced by other biocontrol agents such as *E. cloacae.* Strains of *E. cloacae* with biological control activity produce an unidentified catechol, and many of these strains also produce aerobactin, a hydroxamate siderophore (Loper *et al.* 1993). In a previous study, genes encoding the biosynthesis of aerobactin by *E. cloacae* strain EcCT-501 were cloned, and mutants deficient in aerobactin production (Iuc$^-$) were derived and shown to be similar to the parental strain in suppressing Pythium damping-off of cucumber (Loper *et al.* 1993). The contribution of a putative catechol siderophore produced by *E. cloacae* to the suppression of Pythium damping-off has not been evaluated.

The catechol siderophore enterobactin (also called enterochelin) is commonly produced by species classified in the Enterobacteriaceae, including *Escherichia coli* and clinical isolates of *E. cloacae.* Enterobactin is a cyclic triester of 2,3-dihydroxybenzoyl serine, which is formed by condensation of L-serine and 2,3-dihydroxybenzoic acid (DHBA). The products of the enterobactin biosynthesis genes *entC, entB,* and *entA* catalyze the sequential conversion of chorismic acid to DHBA, an intermediate in the enterobactin biosynthesis pathway. The EntB protein may be bifunctional, because an activity in enterobactin synthesis from DHBA and serine, previously assigned to an *entG* locus, is encoded by the *entB* 3′ terminus (Staab and Earhart 1990). The *entE* product activates the carboxyl group of DHBA for the final steps of enterobactin synthesis. Products of the *entD* and *entF* genes function in the conversion of activated DHBA to enterobactin (Crosa 1989). In *E. coli,* genes encoding enterobactin biosynthesis, ferric enterobactin uptake (*fep*), and ferric enterobac-

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MPMI Vol. 7, No. 4, 1994, pp. 440-448

tin esterase (*fes*) are clustered on the chromosome, spanning approximately 22 kb of DNA in the following order: *entD, fepA, fes, entF, fepE, fepC, fepG, fepD, fepB, entC, entE, entB, entA,* and *P15* (Crosa 1989; Liu *et al.* 1989; Nahlik *et al.* 1989; Pickett *et al.* 1984; Ozenberger *et al.* 1989). These genes are coordinately expressed as four transcripts originating from two bidirectional iron-regulated control regions which contain promoter sequences and ferric uptake regulator (Fur) protein binding sites.

Complementation of enterobactin biosynthesis mutants of *E. coli* has been successfully used to identify and clone genes involved in the biosynthesis of catechol siderophores from several bacterial genera in the Enterobacteriaceae (Bull *et al.* 1994; Enard *et al.* 1991; Massad *et al.*, in press; Schmitt and Payne 1988). In this study, a genomic region of *E. cloacae* that complemented *ent* mutations of *E. coli* was isolated, and mutants of *E. cloacae* that were deficient in catechol siderophore production were derived by introducing a deletion into the genomic *entA* region. The catechol siderophore produced by *E. cloacae* EcCT-501 was preliminarily identified as enterobactin. The individual and combined roles of aerobactin and enterobactin in iron nutrition and biological control activity of *E. cloacae* strain EcCT-501 against *P. ultimum* were evaluated.

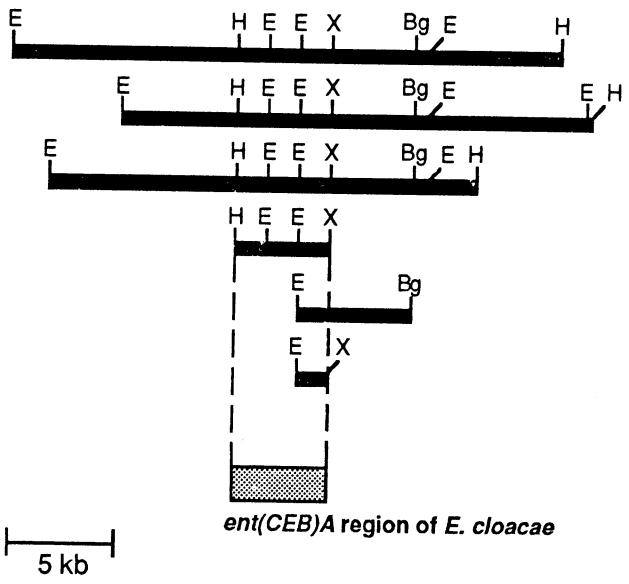
RESULTS

Characterization of the catechol siderophore produced by *E. cloacae* EcCT-501.

E. cloacae strain EcCT-501 produced a catechol in Tris minimal salts (TMS) medium containing 0.1 μM $FeCl_3$ but not in TMS medium containing 100 μM $FeCl_3$. Catechol was extracted from culture supernatants of *E. cloacae* and *E. coli* with ethyl acetate and precipitated with hexane. The catechol extracted from cultures of *E. cloacae* had the same R_f (0.4) as enterobactin extracted from cultures of *E. coli* upon paper chromatography with ammonium formate solvent. The red ferric-catechol complex obtained after the addition of $FeCl_3$ to the catechol extracted from cultures of *E. cloacae* migrated to the same R_f (0.6) as ferric enterobactin on silica gel thin-layer chromatography plates developed in chloroform and methanol (6:5, v/v) (Harris *et al.* 1979b). The absorption maxima of the red ferric-catechol complex were 240, 311, and 495 nm, which are similar to the published values for ferric enterobactin (Van Tiel–Menkveld *et al.* 1982; O'Brien *et al.* 1971). *E. cloacae* strain EcCT-501 cross-fed *E. coli* strain AN93 and *Salmonella typhimurium* strain *enb-1,* which are cross-fed only by strains that produce enterobactin or closely related compounds (Pollack *et al.* 1970).

Identification and cloning of enterobactin biosynthesis genes of *E. cloacae* EcCT-501.

Cosmids containing enterobactin biosynthesis genes were identified by complementation of the *entA403* mutation of *E. coli* from a genomic library of *E. cloacae* EcCT-501 (Loper *et al.* 1993). Nine of the 1,039 cosmids conferred siderophore production on the $EntA^-$ strain AN193, as indicated by the presence of orange halos surrounding colonies on an agar medium containing chrome azurol S (CAS) and by the production of a catechol, as determined by the method of Arnow (1937). The nine cosmids spanned 26 kb of DNA, the limits of which were defined by cosmids pJEL1826 and pJEL1828 (Fig. 1). Two *Eco*RI fragments, of 1.5 and 6.0 kb, that were present in each of the nine cosmids hybridized to the



Complementation of Mutations

	entF	*entC*	*entE*	*entB*	*entA*
pJEL 1828	+	+	+	+	+
pJEL 1826	—	+	+	+	+
pJEL1823	—	+	+	+	+
pJEL 5532	NT	+	+	+	+
pJEL 1870	NT	—	—	—	+
pJEL 1971	NT	—	—	—	+

Fig. 1. Restriction maps of plasmids containing enterobactin biosynthesis genes from *Enterobacter cloacae* strain EcCT-501 and complementation of *ent* mutations of *Escherichia coli*. The *ent(CEB)A* region of *E. cloacae* EcCT-501 is indicated by a speckled bar. pJEL1828, pJEL1826, and pJEL1823 are cosmids constructed in pLAFR3; the *Eco*RI and *Hin*dIII restriction sites shown on the extreme left and right of the inserts were from the polylinker of pLAFR3. The other plasmids contain regions originating from the three cosmids that were subcloned in pUC8. Restoration of the Ent^+ phenotype was tested by chrome azurol S (CAS) agar plate assays (Schwyn and Neilands 1987), cross-feeding bioassays, and the Arnow assay (Arnow 1937). Bg = *Bgl*II; E = *Eco*RI; H = *Hin*dIII; X = *Xho*I; NT = not tested.

entCEBA gene cluster of *E. coli*. Southern analysis with the *entCEBA* region of *E. coli* as a probe detected the two *Eco*RI fragments and also a third hybridizing *Eco*RI fragment, of 3.2 kb, in genomic DNA of *E. cloacae*. The potential involvement of the third genomic *Eco*RI fragment in enterobactin production by *E. cloacae* was not investigated.

Complementation of Ent⁻ mutants of *E. coli* and *S. typhimurium*.

Cosmids pJEL1828, pJEL1826, and pJEL1823 complemented the *entA403, entB402, entC147,* and *entE405* mutations of *E. coli* (Fig. 1) and the *enb-1* mutation of *S. typhimurium,* all of which disrupt enterobactin biosynthesis. Plasmid pJEL1828 also complemented the *entF* mutation of *E. coli*. Therefore, the *entF* homolog of *E. cloacae* was localized to the left of the *ent(CEB)A* homologs, as diagrammed in Figure 1. Neither pJEL1826 nor pJEL1828 complemented the *entD* mutation of *E. coli*. Specific enterobactin biosynthesis gene homologs of *E. cloacae* were further localized by subcloning specific restriction fragments. The 4.5-kb *Hin*dIII-*Xho*I fragment cloned in pJEL5532 complemented the *entA403, entB402, entC147,* and *entE405* mutations of *E. coli* (Fig. 1), indicating the presence of homologs of these four genes on this fragment. The *entA* homolog was further localized to a 1.8-kb *Eco*RI-*Xho*I fragment cloned in pJEL1971. Therefore, the *entA* homolog was located to the right of the *ent(CEB)* homologs, as illustrated in Figure 1. The relative order of the *entC, entE,* and *entB* homologs was not determined.

Derivation of Ent⁻ and Ent⁻ Iuc⁻ mutants of *E. cloacae*.

Sequences within the enterobactin region were deleted from the genome of *E. cloacae* by the marker exchange-eviction mutagenesis technique of Ried and Collmer (1987). A 3.8-kb *Sal*I fragment containing the *nptI-sacB-sacR* cartridge was cloned into the unique *Xho*I site of pJEL1870 to obtain pJEL1969, an *entA* derivative of the unstable plasmid pLAFR3 (Fig. 2). From plasmid pJEL1970, which contains a functional *entA*⁺ homolog, a 0.8-kb *Eco*RV fragment was deleted; the resulting *Eco*RI-*Bam*HI fragment was cloned into pLAFR3 to derive pJEL1973, an unstable plasmid, which did not complement the *entA403* mutation (Fig. 2). The cartridge-containing region of pJEL1969 was exchanged into JL1157, a rifampicin-resistant derivative of strain EcCT-501, and LA122, an Iuc⁻ derivative of JL1157, to produce LA268 and LA269, respectively. The cartridge was then evicted from LA268 and LA269 by exchange substitution of the sequences in pJEL1973, to produce the Ent⁻ strain LA266 and the Ent⁻ Iuc⁻ strain LA235 (Fig. 2). Southern analysis of restriction enzyme–digested DNA probed with pJEL1969 confirmed that insertions and deletions in the genomic DNA of JL1157 and LA122 were as described in Figure 2 (data not shown).

Characterization of Ent⁻ and Ent⁻ Iuc⁻ derivatives of *E. cloacae*.

Strains LA266 (Ent⁻ Iuc⁺) and LA235 (Ent⁻ Iuc⁻) grew at a rate comparable to that of parental strain EcCT-501 in Luria-Bertani (LB) medium; the generation times for all strains ranged between 23 and 24 min. Cosmid pJEL1826, which

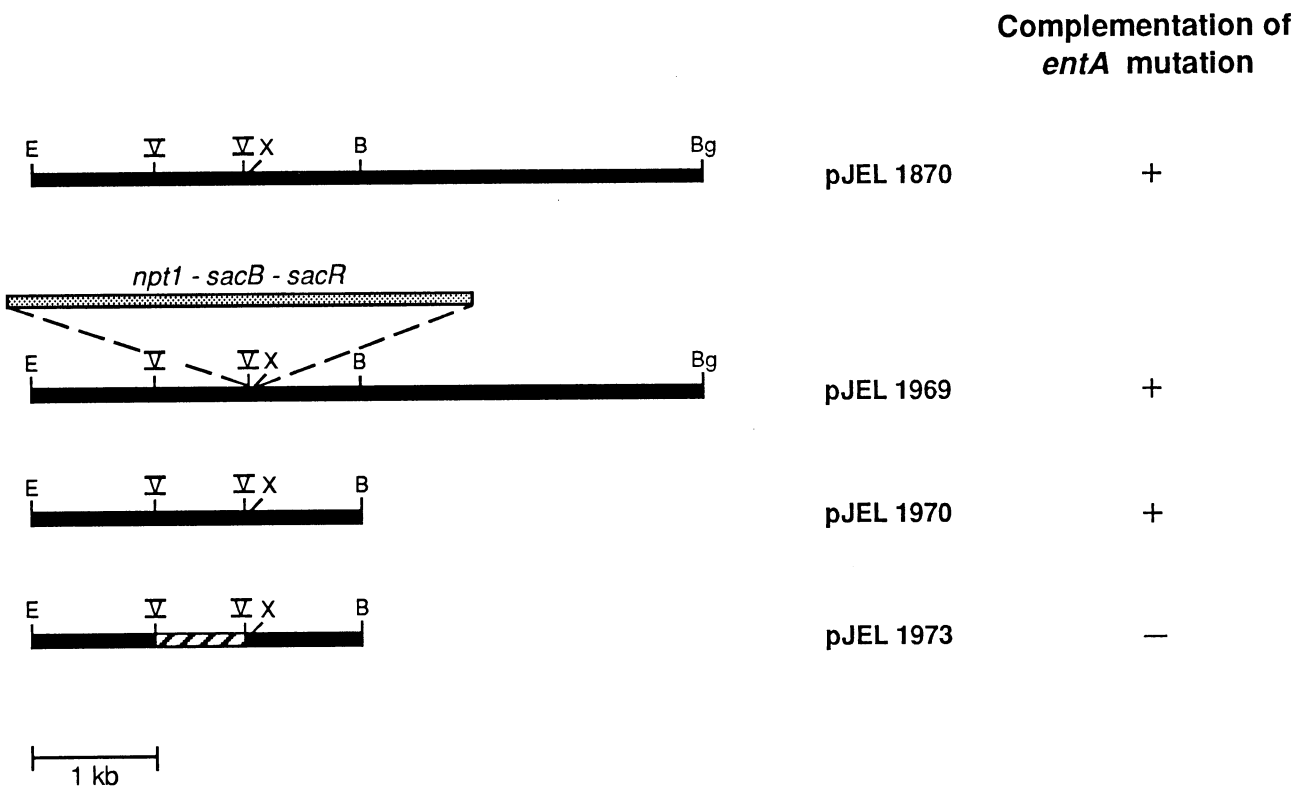


Fig. 2. Restriction maps of plasmids used to construct the Ent⁻ mutants of *Enterobacter cloacae* strain EcCT-501 and complementation of the *entA* mutation of *Escherichia coli*. The dashed lines denote the insertion of the *nptI-sacB-sacR* cartridge into the unique *Xho*I site of pJEL1870 to create pJEL1969. Plasmid pJEL1973 was derived from pJEL1970 by the deletion of an internal *Eco*RV fragment (the deletion is indicated by the bar with diagonal lines). Restoration of the EntA⁺ phenotype was tested by chrome azurol S (CAS) agar plate assays, cross-feeding bioassays, and the Arnow assay (Arnow 1937). B = *Bam*HI; Bg = *Bgl*II; E = *Eco*RI; V = *Eco*RV; X = *Xho*I.

contains the cloned enterobactin biosynthesis genes of EcCT-501 (Fig. 1), restored enterobactin production to both LA235 and LA266. Therefore, a role of the cloned region in enterobactin production by strain EcCT-501 was confirmed. Ent^+ Iuc^+, Ent^- Iuc^+, and Ent^+ Iuc^- strains grew on an iron-deficient medium (TMS medium containing up to 250 μM 2,2′-dipyridyl), whereas an Ent^- Iuc^- strain did not grow on this medium; thus, both enterobactin and aerobactin functioned in iron acquisition by *E. cloacae*.

Biological control of Pythium damping-off of cucumber and cotton by *E. cloacae* and derivatives deficient in siderophore production.

E. cloacae EcCT-501, and its Ent^- Iuc^+, Ent^+ Iuc^-, and Ent^- Iuc^- derivatives were effective agents for the biological control of Pythium damping-off of cucumber. Seed treatment with any of the four strains increased seedling emergence and the proportion of healthy seedlings relative to seed treatment with water only (Table 1). The four strains did not differ in population sizes established on root systems of cucumber or cotton seedlings (the mean rhizosphere population size was 5.4 $\log_{10}$[colony-forming units per root system] at 10 days after planting).

DISCUSSION

This study established the presence of two siderophores in the biological control agent *E. cloacae* strain EcCT-501: the hydroxamate aerobactin (Loper *et al.* 1993) and a catechol, which was tentatively identified as enterobactin, on the basis of its extractability in ethyl acetate, migration on thin-layer chromatography plates, absorbance spectra, and capacity to cross-feed strains of *E. coli* that utilize ferric enterobactin as an iron source. Mutants derived from EcCT-501 that produced either aerobactin (Ent^- Iuc^+) or enterobactin (Ent^+ Iuc^-) grew on an iron-limited medium, whereas a mutant deficient in the production of both siderophores (Ent^- Iuc^-) did not grow under iron-limiting conditions. Thus, either ferric aerobactin or ferric enterobactin provided iron for bacterial growth in low-iron environments. There was no clear functional superiority observed in the siderophores produced by *E. cloacae:* Ent^- Iuc^+ and Ent^+ Iuc^- mutants grew similarly under conditions of iron depletion. This result was surprising, because ferric enterobactin, having a stability constant of 10^{52} (Harris *et al.* 1979b), has a far greater affinity for the ferric ion than does ferric aerobactin, having a stability constant of 10^{23} (Harris *et al.* 1979a). In addition, enterobactin is superior to aerobactin for iron acquisition by strains of *Shigella flexneri* grown in culture (Payne *et al.* 1983). Nevertheless, aerobactin rather than enterobactin provides a selective advantage for bacterial growth in extracellular compartments of mammalian tissues or in serum, where the ferric ion is sequestered primarily by the iron-binding glycoprotein transferrin. Aerobactin is relatively stable, active over a range of pH values, and secreted during the early stages of bacterial cell growth, and it can be recycled, whereas enterobactin is degraded following transport into the bacterial cell (Payne 1988). A differential role for aerobactin or enterobactin in iron acquisition by *E. cloacae* may be detected in future studies evaluating the contribution of these compounds to the ecological fitness of the bacterium in natural habitats.

Neither aerobactin nor enterobactin production contributed to the suppression of Pythium damping-off of cucumber or cotton by *E. cloacae* strain EcCT-501. Biological control of Pythium damping-off was evaluated in soils of neutral pH and low iron content, in which iron availability was expected to be sufficiently low for siderophore-mediated iron competition to occur. The biological control activity of the Ent^- Iuc^- mutant of *E. cloacae* could not be attributed to the known production of another siderophore, because it did not produce other siderophores in culture (on CAS agar). Nevertheless, the possibility exists that *E. cloacae* produces siderophores in the plant rhizosphere that are not produced in culture. This study supports the conclusions of a previous report that aerobactin production contributes little to the suppression of Pythium damping-off of cucumber by *E. cloacae* (Loper *et al.* 1993). The results of these and other recent studies (Kraus and Loper 1992; Paulitz and Loper 1991) indicate that

Table 1. Efficacy of *Enterobacter cloacae* EcCT-501 and derivatives in biological control of Pythium damping-off of cucumber and cotton

		Cucumber[a]		Cotton[a]	
Treatment	Phenotype[b]	Emergence (%)[c]	Healthy seedlings (%)[d]	Emergence (%)[c]	Healthy seedlings (%)[d]
Steamed soil	Ent^+ Iuc^+	92 a	92 a	100 a	100 a
JL1157	Ent^- Iuc^+	69 bc	56 b	71 c	60 b
LA266	Ent^- Iuc^-	83 ab	59 b	83 bc	69 b
LA235	Ent^+ Iuc^-	74 bc	61 b	83 bc	69 b
LA122		67 c	58 b	83 bc	69 b
Control (untreated)		50 d	32 c	40 d	21 c
Metalaxyl		NT	NT	93 ab	93 ab
$LSD_{0.05}$		15.9	16.7	16.3	18.7

[a] Means in the same column that are followed by the same letter do not differ statistically ($P = 0.05$) by Fisher's protected least significant difference test. The data are mean values from a representative experiment. Cucumber and cotton seeds were dipped in bacterial suspensions or in sterile water (untreated control) before being planted in soil infested with 100 propagules of *Pythium ultimum* per gram ($n = 12$). NT = not tested.

[b] Iuc^+ = aerobactin production based on hydroxamate production and cross-feeding of aerobactin-utilizing strains of *Escherichia coli* (Loper *et al.* 1993). Iuc^- = no aerobactin production. Ent^+ = enterobactin production as determined by the Arnow assay (Arnow 1937) and cross-feeding assays of enterobactin-utilizing strains of *E. coli*. Ent^- = no enterobactin production.

[c] Percentage of seedlings that did not succumb to preemergence Pythium damping-off.

[d] Percentage of seedlings that did not succumb to preemergence and postemergence damping-off caused by *Pythium ultimum*.

siderophore production is not a dominant mechanism by which rhizosphere bacteria suppress Pythium damping-off of cucumber. Pyoverdine production, however, is critical to suppression of Pythium damping-off of cotton by *P. fluorescens* (Loper 1988). In contrast to pyoverdine production by *P. fluorescens*, aerobactin and enterobactin production by *E. cloacae* did not contribute to suppression of Pythium damping-off of cotton.

Virtually every plant pathogen, symbiont, or saprophyte that has been evaluated produces siderophores (Loper and Buyer 1991). Nevertheless, investigations of the importance of siderophores as factors contributing to the fitness or antagonistic properties of biological control agents have focused almost exclusively on pyoverdine siderophores produced by fluorescent pseudomonads. Ferric pyoverdines, with stability constants of 10^{32} (Meyer and Abdallah 1978), have affinities for iron that are intermediate to those of ferric enterobactin and ferric aerobactin. The relative proficiency of these siderophores to sequester iron in the rhizosphere depends theoretically on their relative concentrations, equilibrium constants in the chemical environment of the rhizosphere, and the kinetics of association (Loper and Buyer 1991). Because these factors are not readily predicted, however, the importance of siderophores to biological control activity must be determined em-

Table 2. Bacterial strains and plasmids used in this study

	Description	Relevant characteristics[a]	Source or reference
Escherichia coli			
DH5α	*F⁻, endA1, hsdR17*(r_k^-,m_k^+) *supE44, thi-1, recA1, gyrA96, relA1, φ80dlacZΔ*, M15, λ⁻	Ent⁺	Bethesda Research Laboratories
AN90	*F⁻, tonA23, procC14, leu16, trpE38, thi-1, entD*	EntD⁻, Sm^r	J. B. Neilands
AN93	*F⁻, tonA23, procC14, leu16, trpE38, thi-1, entE405*	EntE⁻, Sm^r	J. B. Neilands
AN102	*leu16, proC14, trpE38, thi-1, fepA*	FepA⁻	J. B. Neilands
AN117	*F⁻, tonA23, procC14, leu16, trpE38, thi-1, entF*	EntF⁻, Sm^r	J. B. Neilands
AN192	*F⁻, tonA23, procC14, leu16, trpE38, thi-1, entB402*	EntB⁻, Sm^r	J. B. Neilands
AN193	*F⁻, tonA23, procC14, leu16, trpE38, thi-1, entA403*	EntA⁻, Sm^r	J. B. Neilands
MT147	*entC147*::*Km* derivative of AB1515	EntC⁻, Km^r	Ozenberger *et al.* 1989
Salmonella typhimurium			
enb-1	Derivative of LT-2, *asc-1*	Ent⁻, Sm^r	Pollack *et al.* 1970
Enterobacter cloacae			
EcCT-501	Biological control agent	Ent⁺, Iuc⁺	Isolated from cotton hypocotyl (Nelson 1988)
JL1157	Rif^r derivative of EcCT-501	Ent⁺, Iuc⁺	Loper *et al.* 1993
LA122	Derivative of JL1157, Δ(*iuc*)	Ent⁺, Iuc⁻	Loper *et al.* 1993
LA268	Derivative of JL1157, *ent*::*nptI-sacB-sacR*	Ent⁺, Iuc⁺, Km^r, sucrose sensitivity, Rif^r	This study
LA269	Derivative of LA122, Δ(*iuc*), *ent*::*nptI-sacB-sacR*	Ent⁺, Iuc⁻, Rif^r	This study
LA235	Derivative of LA269, Δ(*ent*), Δ(*iuc*)	Ent⁻, Iuc⁻, Rif^r	This study
LA266	Derivative of LA268, Δ(*ent*)	Ent⁻, Iuc⁺, Rif^r	This study
LA270	LA235 (pJEL1826)	Ent⁺, Iuc⁺, Rif^r	This study
LA271	LA266 (pJEL1826)	Ent⁺, Iuc⁺, Rif^r	This study
Plasmids			
pCP410	(*entCEBA*)⁺ genes of *E. coli*, 6.7-kb *Eco*RI fragment cloned in pACYC184	EntCEBA⁺, Tc^r	Pickett *et al.* 1984
pUC8	ColE1 replicon	Ap^r	Vieira and Messing 1982
pLAFR3	*cos, inc*P1 replicon, polylinker of pUC8	Tc^r, Tra⁻, Mob⁺	Staskawicz *et al.* 1987
pRK2013	Mobilizing plasmid	Tra⁺, Km^r	Figurski and Helinski 1979
pUM24	pUC4K derivative containing *nptI-sacB-sacR* cartridge	Km^r, Ap^r, sucrose sensitivity	Ried and Collmer 1987
pJEL1574	18.7-kb fragment from EcCT-501 cloned into pLAFR3	Iuc⁺, Iut⁺, Tc^r	Loper *et al.* 1993
pJEL1823	21.4-kb fragment from EcCT-501 cloned into pLAFR3	Ent(CEB)A⁺, Tc^r	This study
pJEL1826	22.4-kb fragment from EcCT-501 cloned into pLAFR3	Ent(CEB)A⁺, Tc^r	This study
pJEL1828	25.8-kb fragment from EcCT-501 cloned into pLAFR3	Ent(CEB)AF⁺, Tc^r	This study
pJEL1870	5.5-kb *Eco*RI-*Bgl*II fragment of pJEL1862 cloned into pLAFR3	EntA⁺, Tc^r	This study
pJEL1969	*nptI-sacB-sacR* cartridge cloned into *Xho*I site of 5.5-kb *Eco*RI-*Bgl*II fragment of pJEL1870	EntA⁺, Tc^r, Km^r	This study
pJEL1970	2.7-kb *Eco*RI-*Bam*HI fragment of pJEL1862 cloned into pUC8	EntA⁺, Ap^r	This study
pJEL1971	1.8-kb *Eco*RI-*Xho*I fragment of pJEL1862 cloned into pUC8	EntA⁺, Ap^r	This study
pJEL1973	pJEL1970 with an internal *Eco*RV deletion	EntA⁻, Tc^r	This study
pJEL5532	4.5-kb *Hin*dIII-*Xho*I fragment of pJEL1990 cloned into pUC8	Ent(CEB)A⁺, Ap^r	This study

[a] Ap^r, ampicillin resistance; Ent⁺ and Ent⁻, enterobactin producer and nonproducer, respectively; FepA⁻, lack of outer-membrane ferric enterobactin receptor protein; Iuc⁺ and Iuc⁻, aerobactin producer and nonproducer, respectively; Iut⁺, presence of ferric aerobactin receptor; Km^r, kanamycin resistance; Mob⁺, mobilizable plasmid; Rif^r, rifampicin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; Tra⁺ and Tra⁻, self-transmissible and non-self-transmissible plasmids, respectively.

pirically. In this study, siderophores produced by strains of *E. cloacae* were characterized genetically and evaluated as factors potentially contributing to the antagonistic properties of this bacterium. Although aerobactin and enterobactin production by *E. cloacae* contributed little to biological control activity against *P. ultimum,* it may contribute to the ecological fitness of *E. cloacae* and its interactions with other rhizosphere and spermosphere microorganisms.

Enterobactin biosynthesis genes appear highly conserved in strains of *E. coli, Salmonella typhimurium,* and *Shigella flexneri* that produce this siderophore (Faundez *et al.* 1990; Schmitt and Payne 1988). Genes encoding enzymes catalyzing biosynthetic steps resulting in the production and activation of DHBA are clustered in a single operon with the order *entCEBA*. Although the relative order of the *entC, entE,* and *entB* gene homologs of *E. cloacae* strain EcCT-501 was not resolved, the genes were localized to a common 4.5-kb *Hin*dIII-*Xho*I fragment approximately 5.5 kb from an *entF* homolog in the genome of *E. cloacae*. Genomic DNA complementing the *entA* mutation was localized to a 1.8-kb *Eco*RI-*Xho*I fragment within the 4.5-kb fragment, providing evidence for a gene order of *entF(CEB)A*. Enterobactin biosynthesis genes of *E. cloacae* strain EcCT-501 hybridized to those of *entCEBA* of *E. coli* (Fig. 1), providing further evidence for the similarity of the DHBA biosynthesis regions in enterobactin-producing strains of the Enterobacteriaceae.

MATERIALS AND METHODS

Bacterial strains and media.

Bacterial strains and plasmids are listed in Table 2. *E. coli* and *E. cloacae* were cultured on LB medium (Sambrook *et al.* 1989) at 35° C. Growth rates of strains of *E. cloacae* were determined by changes in OD_{600} of cultures grown with shaking (200 rpm) at 35° C in LB broth. In some cloning experiments, LB agar was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml) (International Biotechnologies, New Haven, CT) and isopropyl β-D-thiogalactopyranoside (IPTG) (100 μg/ml) (Sigma Chemical Company, St. Louis, MO) for screening transformants. Antibiotics (Sigma) were used at the following concentrations: ampicillin (100 μg/ml), kanamycin (50 μg/ml), rifampicin (100 μg/ml), and tetracycline (20 μg/ml), except where different concentrations are specified.

Siderophores were produced in TMS medium (Simon and Tessman 1963) supplemented with casamino acids (0.3%), tryptophan (0.003%), and thiamine (0.002%). Stock solutions of casamino acids were extracted with 8-hydroxyquinoline and chloroform (Bell *et al.* 1979) to remove contaminating iron. For growth under iron-limited conditions, strains of *E. cloacae* were grown overnight at 35° C with shaking (200 rpm) in TMS broth amended with 0.1 μM $FeCl_3$. A 3-μl sample of the culture was then spotted onto the surface of TMS agar supplemented with 50, 100, 150, 200, or 250 μM 2,2′-dipyridyl. The plates were incubated at 35° C and observed 24 and 48 hr after inoculation for bacterial growth.

Detection of siderophore production.

Siderophore production was detected by observing orange halos surrounding test strains grown on CAS agar (Schwyn and Neilands 1987). Catechols in supernatants of cultures grown for 24–48 hr in TMS were detected by the methods of Arnow (1937). Hydroxamates in culture supernatants were detected by the methods of Atkin *et al.* (1970).

Partial purification and chromatography of a catechol produced by *E. cloacae*.

For production of the catechol, 5-ml samples of an overnight culture of *E. cloacae* or *E. coli* AN102 were inoculated into 1-L flasks, each containing 200 ml of the liquid medium described by Neilands and Nakamura (1991). The flasks were incubated on a rotary shaker at 35° C for 20 hr, after which cells were removed by centrifugation. Enterobactin was extracted from culture supernatants with ethyl acetate washed with 0.1M citrate buffer, pH 5.5 (Neilands and Nakamura 1991). The catechol in supernatants, ethyl acetate extracts, and fractions from purification steps was detected by the assay of Arnow (1937). Arnow-positive fractions were evaluated by thin-layer chromatography developed in chloroform and methanol (6:5, v/v) (Harris *et al.* 1979b) and paper chromatography developed in 0.5% formic acid and 5% ammonium formate (v/v) (Van Tiel-Menkveld *et al.* 1982). $FeCl_3$ (100 μl, 0.001 M) was added to 100 μl of an aqueous solution of the catechol siderophore, and absorbance was measured with a Shimadzu spectrophotometer (Shimadzu, Kyoto, Japan).

Enterobactin bioassay.

Enterobactin production by bacterial strains was detected by cross-feeding of the indicator strains *E. coli* AN93 and *S. typhimurium* enb-1, which are unable to grow under iron-limiting conditions unless provided with enterobactin or a related compound (Pollack *et al.* 1970). Molten TMS agar containing 150 μM 2,2′-dipyridyl was seeded with approximately 10^6 cfu of the indicator strain per milliliter. Strains evaluated for enterobactin production were spotted onto the surface of solidified seeded TMS medium and incubated at 35° C. After 24–48 hr, growth of the indicator strain around colonies indicated production of enterobactin.

Nucleic acid methods.

Plasmids were isolated by an alkaline lysis procedure (Sambrook *et al.* 1989) 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 (CTAB) in chloroform (Ausubel *et al.* 1987), prior to standard phenol:chloroform extraction and ethanol precipitation. Electrophoresis was in 0.5–1.0% agarose gels with Tris-phosphate-EDTA (TPE) buffer (Sambrook *et al.* 1989). Ligations, alkaline phosphatase treatments, restriction endonuclease digestions, and transformation procedures were performed using standard procedures (Sambrook *et al.* 1989).

Southern hybridizations.

Probes for Southern blots were 1) the *entCEBA* region of *E. coli,* a 6.7-kb *Eco*RI fragment of pCP410 (Pickett *et al.* 1984), and 2) cosmid pJEL1969, which contains a 5.5-kb genomic region of *E. cloacae* and the *nptI-sacB-sacR* cartridge. Restriction fragments used as a probe were purified from agarose gels (SeaKem GTG, FMC BioProducts, Rockland, ME)

by adsorption and elution from NA-45 DEAE membranes, according to the manufacturer's recommendations (Schleicher and Schuell, Keene, NH). DNA probes were labeled by random hexamer primer labeling with [^{32}P]dCTP according to recommendations of the manufacturer (Gibco BRL Life Technologies, Gaithersburg, MD). The ^{32}P-labeled probes were purified with a Nu-clean D-50 column (International Biotechnologies, New Haven, CT). Hybridization conditions were moderately stringent (42° C, 50% formamide, and 0.16× SSC) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the subsequent washes were in 0.1× SSC at 55° C (Sambrook *et al.* 1989).

Mobilization of plasmids.

Cosmid pLAFR3 and derivatives were mobilized from *E. coli* strain DH5α into rifampicin-resistant derivatives of *E. cloacae* or Ent$^-$ mutants of *E. coli* by triparental matings with pRK2013 as a helper plasmid, or from *E. coli* strain S17-1 (Simon *et al.* 1983) in biparental matings with rifampicin-resistant derivatives of *E. cloacae* as recipient. Transconjugants (*E. cloacae* harboring pLAFR3 derivatives) were selected on LB agar amended with tetracycline (60 μg/ml) and rifampicin. Transconjugants were typically obtained at a frequency of approximately 10^{-1} transconjugant per donor. Plasmid pUC8 and derivatives were introduced into Ent$^-$ strains of *E. coli* by transformation.

Identification of enterobactin biosynthesis genes of *E. cloacae*.

An existing library of EcCT-501 genomic DNA (Loper *et al.* 1993), constructed in the cosmid vector pLAFR3, was mobilized into the EntA$^-$ strain of *E. coli* AN193. Approximately 1,000 transconjugants were screened for siderophore production on CAS agar (Schwyn and Neilands, 1987). Colonies that produced an orange halo on CAS agar were further tested for catechol and enterobactin as described above.

Derivation of Ent$^-$ and Ent$^-$ Iuc$^-$ derivatives of *E. cloacae*.

The marker exchange-eviction mutagenesis technique of Ried and Collmer (1987) was used to construct directed, unmarked mutations in JL1157, a rifampicin-resistant derivative of *E. cloacae* strain EcCT-501. The *nptI-sacB-sacR* cartridge, which is carried on a 3.8-kb *Bam*HI fragment of pUM24 (Ried and Collmer 1987), confers kanamycin resistance on *E. cloacae,* due to *nptI,* and sucrose sensitivity, due to the production of levan sucrase conferred by *sacB*. *E. cloacae* grew on 925 agar medium (Langley and Kado 1972), a minimal medium containing 10% sucrose as the 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 that resulted in insertion of the cartridge into the bacterial genome were selected on LB medium amended with kanamycin; those resulting in eviction of the cartridge from the genome were selected on 925 containing 10% sucrose (Loper *et al.* 1993). Cultures of EcCT-501 containing pLAFR3 derivatives were grown at 35° C with shaking in 200 ml of LB broth in the absence of tetracycline. After 10–24 hr, 0.1 ml of culture was transferred to 200 ml of fresh LB medium. After four or five successive transfers, pLAFR3 derivatives were lost from more than 90% of bacterial cells.

Biological control tests.

Warden sandy silt loam soil was used for all experiments. The soil, pH 7.7, contained 1.02% organic matter, phosphorus at 13 mg/kg, potassium at 250 mg/kg, iron at 5 mg/kg, and calcium at 176 meq/kg. Inoculum of *P. ultimum* for infestation of soil was prepared as described by Paulitz and Baker (1987) and Loper *et al.* (1993). Steamed soil was infested with 100 propagules of *P. ultimum* per gram of soil. For the cucumber experiments, infested soil was placed in cells 3.5 cm in diameter and 4 cm deep in plastic trays (TLC Polyform, Plymouth, MN) and amended with a volume of deionized water to bring the final soil moisture to −0.01 MPa. The trays were covered with plastic wrap and incubated in a growth chamber at 24° C for 3 days, to allow the soil moisture to equilibrate. For the cotton experiments, soil was placed in plastic containers 4 cm in diameter and 20.5 cm deep (Stuewe & Sons, Corvallis, OR). Bacterial cells grown overnight in LB broth were centrifuged and resuspended in water to a final concentration of 10^8 cfu/ml. Seeds of cucumber (cultivar Marketmore) and cotton (cultivar Stoneville 213) were soaked in bacterial suspensions for 10 min prior to planting. Each treatment had 12 replications, consisting of seven cells (cucumber) or containers (cotton), in each of which an individual seed was sown. The cells and containers were placed in growth chambers maintained at 20° C. Percent emergence and percent healthy seedlings were scored 10 days after planting. The mean percent emergence and percent healthy seedlings of the seven plants within a replication were used for statistical analyses. Rhizosphere population sizes of rifampicin-resistant bacteria were estimated from the entire root systems of 12 replicate plants per treatment by dilution plating of root washings on LB amended with rifampicin (100 μg/ml) and cycloheximide (50 μg/ml) by published methods (Paulitz and Loper 1991). The cotton and cucumber experiments were performed at least twice.

Data analysis.

Pythium damping-off data and bacterial rhizosphere population data for bacterial treatments were analyzed by the analysis of variance procedure of Statistical Analysis Systems (SAS Institute, Cary, NC). The logarithmic (base 10) transformation was applied to individual estimations of rhizosphere bacterial population size prior to analysis. Fisher's protected least significant difference was used for mean separation for the damping-off and bacterial population data (Fisher 1966).

ACKNOWLEDGMENTS

We thank Jennifer Kron for assistance in the derivation of mutant strains, their characterization, and biological control assays; Marcella Henkels for assistance in biological control experiments and drawing figures; Kristen Hoffbuhr for assistance in complementation studies; Greg Boyer and Brian Nowak-Thompson for assistance in enterobactin identification; Alan Collmer, C. F. Earhart, Mark McIntosh, J. B. Neilands, and Eric Nelson for providing strains; Susan Carnegie for excellent technical assistance; and Mike Boehm, Carol Ishimaru, Jennifer Kraus, and Virginia Stockwell for critical reviews of this manuscript. This research was supported in part by grant number 91-37030-6802 from the U.S. Department of Agriculture, Competitive Grants Program.

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