Cloning and Characterization of Aerobactin Biosynthesis Genes of the Biological Control Agent *Enterobacter cloacae*

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Five strains of *Enterobacter cloacae* that are biological control agents of *Pythium* damping-off diseases produced the hydroxamate siderophore aerobactin under iron-limiting conditions. Genes determining aerobactin biosynthesis of the biocontrol strain *E. cloacae* EcCT-501 were localized to a 12.3-kb region, which conferred aerobactin production to *Escherichia coli* DH5 α . The aerobactin biosynthesis genes of *E. cloacae* hybridized to those of the pColV-K30 plasmid of *E. coli*, but restriction patterns of the aerobactin regions of pColV-K30 and *E. cloacae* differed. A derivative strain with a deletion in the aerobactin biosynthesis locus was as effective as strain EcCT-501 in biological control of *Pythium* damping-off of cucumber. Thus, aerobactin production did not contribute significantly to the biological control activity of EcCT-501 under the conditions of this study.

Enterobacter cloacae is widely distributed in nature, where it is commonly associated with a variety of plant and animal species, soil, and water (9). Certain strains of *E. cloacae*, which were isolated from seed, hypocotyl tissue, or the rhizosphere, are effective agents for the biological control of damping-off diseases of various crop plants caused by *Pythium* spp. (18, 34, 38). Although the mechanism(s) by which *E. cloacae* suppresses *Pythium* damping-off is unknown, several hypotheses have been proposed (37). For example, the capacity of *E. cloacae* to catabolize seed exudates that are required for sporangial germination by *Pythium ultimum* may reduce seed infection by *P. ultimum* (35). Second, when grown on certain media, *E. cloacae* deaminates amino acids to produce ammonia at levels that are toxic to *P. ultimum* (18); toxic levels of ammonia also may be generated by *E. cloacae* inhabiting the spermosphere of certain plants. Finally, in paired cultures of *P. ultimum* and *E. cloacae*, bacterial cells adhere firmly to hyphal cell walls, resulting in a reduction in fungal growth (38). The capacity to bind to fungal hyphae also may be a key event in the antagonism of *P. ultimum* by *E. cloacae* in the spermosphere (37).

In addition to *E. cloacae*, certain strains of *Pseudomonas fluorescens* and *Pseudomonas putida* suppress *Pythium* damping-off diseases (4, 13, 19, 20, 23, 27, 39, 41). *Pseudomonas* spp. produce a variety of secondary metabolites, including antibiotics and siderophores, which suppress hyphal growth of *P. ultimum* in culture and in the spermosphere or rhizosphere (16, 19, 20, 28). 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 (33). It is hypothesized that fluorescent siderophores, termed pyoverdines (also pyoverdins or pseudobactins), produced by *Pseudomonas* spp. deplete the microenvironment surrounding a pathogen of

available iron by sequestering ferric ions as ferric-pyoverdine complexes (26, 28, 48). Because *P. ultimum* is sensitive to siderophore-mediated iron competition with *Pseudomonas* spp. (4, 28), we reasoned that it may also be sensitive to iron deprivation imposed by siderophores produced by *E. cloacae*. Preliminary studies suggested that strains of *E. cloacae* with biological control activity produced one or more siderophores (22, 52), as do clinical strains of *E. cloacae* (11, 54).

Many enterobacteria synthesize catechol siderophores, such as enterobactin (also called enterochelin), or hydroxamate siderophores, such as aerobactin (43). Aerobactin was isolated first from cultures of *Aerobacter aerogenes* (15). Other bacterial species within the family Enterobacteriaceae, including *Erwinia carotovora* (21), *Shigella flexneri* (42), *E. cloacae* (11, 54), and *Escherichia coli* (10, 57), also synthesize aerobactin. Aerobactin production is one of several virulence factors of invasive strains of *E. coli* (43), enabling bacterial proliferation in the iron-deficient intercellular environment of mammalian tissues.

Our studies focus on the characterization and biological significance of iron acquisition systems of plant-associated enterobacteria, including the biological control agent *E. cloacae*. In this report, we present evidence for hydroxamate siderophore production by five strains of *E. cloacae* with biological control activity. From one of these strains, we identified the hydroxamate as aerobactin, characterized the genes encoding for the aerobactin iron acquisition system, and derived a mutant deficient in aerobactin production. We also demonstrated that aerobactin production was not required for biological control of *Pythium* damping-off of cucumber by *E. cloacae* EcCT-501.

(An abstract of this research has been published [22].)

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids are listed in Table 1. *E. coli* and *E. cloacae* were cultured routinely on Luria Bertani (LB) medium (47) at 35°C. Growth rates of strains of *E. cloacae* were determined by the change in optical density at 600 nm of cultures grown with

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Relevant characteristics ^a	Source (reference)
<i>Escherichia coli</i>			
DH5 α	F ⁻ <i>endA1 hsdR17</i> (r _k ⁻ , m _k ⁺) <i>supE44 thi-1 recA1 gyrA96 relA1 ϕ80dlacZΔM15 λ⁻</i>	Ent ⁺	Bethesda Research Laboratories
LG1315	<i>ara entA lac leu mtl proC rpsL supE thi tonA trpE xyl</i> (pColV-K30)	Ent ⁻ Iuc ⁺ Iut ⁺	J. H. Crosa (56)
LG1522	<i>ara azi fepA lac leu mtl proC rpsL supE tonA tsx thi iuc</i> (pColV-K30 <i>iuc</i>)	FepA ⁻ Iuc ⁻ Iut ⁺	J. H. Crosa (56)
<i>Enterobacter cloacae</i>			
EcCT-501	Biological control agent	Iuc ⁺	E. B. Nelson; isolated from the cotton hypocotyl (34, 36)
E1	Biological control agent	Iuc ⁺	E. B. Nelson; isolated from cucumber seed (36)
E6	Biological control agent	Iuc ⁺	E. B. Nelson; isolated from cucumber seed (36)
E13	Biological control agent	Iuc ⁺	E. B. Nelson; isolated from cucumber seed
EcH-1	Biological control agent	Iuc ⁺	C. R. Howell; isolated from cotton rhizosphere soil (18)
JL1157	Biological control agent	Iuc ⁺ Rif ^r	Derivative of EcCT-501 with spontaneous resistance to rifampin (this study)
LA115	Derivative of EcCT-501, <i>iuc::nptI-sacB-sacR</i>	Iuc ⁻ Km ^r ; sucrose sensitive	This study
LA121	Derivative of EcCT-501, Δ (<i>iuc</i>)	Iuc ⁻	This study
LA122	Derivative of JL1157, Δ (<i>iuc</i>)	Iuc ⁻ Rif ^r	This study
DF13	Cloacin DF13 producer		J. H. Crosa (54)
Plasmids			
pABN1	(<i>iucABCD</i>) ⁺ , <i>iutA</i> ⁺ genes of pColV-K30 cloned in pPlac	Iuc ⁺ Iut ⁺ Ap ^r	J. B. Neilands (7)
pLAFR3	<i>cos</i> , <i>incP1</i> replicon, polylinker of pUC8	Tc ^r LacZ ⁺ Mob ⁺	B. J. Staskawicz (51)
pRK2013	Mobilizing plasmid	Tra ⁺ Km ^r	14
pUM24	pUC4K derivative containing <i>nptI-sacB-sacR</i> cartridge	Km ^r Ap ^r ; sucrose sensitivity	A. Collmer (45)
pJEL1572	24.4-kb fragment from EcCT-501 cloned into pLAFR3	Iuc ⁺ Iut ⁺ Tc ^r	This study
pJEL1574	18.7-kb fragment from EcCT-501 cloned into pLAFR3	Iuc ⁺ Iut ⁺ Tc ^r	This study
pJEL1576	16.6-kb fragment from EcCT-501 cloned into pLAFR3	Iuc ⁺ Iut ⁺ Tc ^r	This study
pJEL1580	21.2-kb fragment from EcCT-501 cloned into pLAFR3	Iuc ⁺ Iut ⁺ Tc ^r	This study
pJEL1582	21.7-kb fragment from EcCT-501 cloned into pLAFR3	Iuc ⁺ Iut ⁺ Tc ^r	This study
pJEL1584	18.8-kb fragment from EcCT-501 cloned into pLAFR3	Iuc ⁺ Iut ⁺ Tc ^r	This study
pJEL1741	<i>nptI-sacB-sacR</i> cartridge cloned into <i>Bgl</i> II site of 4.4-kb <i>Eco</i> RI fragment of pJEL1574; in pLAFR3	Tc ^r Km ^r ; sucrose sensitivity	This study
pJEL1771	pJEL1574 with deletion of two internal <i>Sst</i> I fragments	Iuc ⁻ Iut ⁺ Tc ^r	This study

^a Ent⁺ and Ent⁻, enterobactin producer and nonproducer, respectively; Nal^r, nalidixic acid resistant; Rif^r, rifampin resistant; Iuc⁺ and Iuc⁻, aerobactin producer and 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.

shaking at 35°C in LB broth. Siderophores were produced in M9 medium (31) or Tris minimal salts medium (TMS) (50) 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 (5) to remove contaminating iron. In some cloning experiments, LB agar was supplemented with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; 40 μ g/ml; International Biotechnologies, Inc., New Haven, Conn.) and isopropyl- β -D-thiogalactopyranoside (IPTG; 100 μ g/ml; Sigma Chemical Co., St. Louis, Mo.) for screening of transformants. Antibiotics (Sigma) were used at the following concentrations: ampicillin (100 μ g/ml), cephalixin (100 μ g/ml), kanamycin (50 μ g/ml), and tetracycline (20 μ g/ml), except where different concentrations are specified.

Detection of siderophore production. Siderophore production was detected by observation of orange halos surround-

ing test strains grown on CAS agar, a medium for detection of high-affinity iron-chelating compounds (49). Catechol(s) was detected from supernatants of cultures grown for 24 to 48 h in TMS by the method of Arnou (1) or Rioux et al. (46). Hydroxamate(s) in culture supernatants was detected by the methods of Atkin et al. (2) and Csáky (12).

Aerobactin bioassay. Molten TMS containing 150 μ M 2,2'-dipyridyl was seeded with approximately 10⁶ CFU of *E. coli* 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 aerobactin-producing colonies.

Purification and characterization of aerobactin. Hydroxamates were isolated, by the method of Gibson and Magrath (15), from five cultures, each grown with shaking (200 rpm) for 42 h at 27°C in a 2-liter flask containing 500 ml of M9

medium supplemented with 50 μ M 2,2'-dipyridyl. Hydroxamates were separated on cellulose thin-layer chromatography plates (MN-300; Analtech, Newark, Del.) in a butanol-acetic acid-water solvent system (60:15:25) 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 on a Bruker WM400 instrument by repeated exchanges in deuterium oxide. Assignment of the methylene protons from hydroxylysine were made by comparison with the spectrum of the unmodified amino acid (58).

Nucleic acid isolation and hybridization. Plasmids were isolated by an alkaline lysis procedure (47) and purified by ethidium bromide-caesium chloride density gradient centrifugation. For isolation of genomic DNA, cells were lysed with sodium lauryl sulfate, treated with proteinase K, and extracted with hexadecyl-trimethylammonium bromide in chloroform (3) prior to standard phenol-chloroform extraction and ethanol precipitation. Electrophoresis was in 0.5 to 1.3% agarose gels with Tris-phosphate EDTA buffer (47). Ligations, alkaline phosphatase treatments, restriction endonuclease digestions, and transformation procedures were standard (47). For Southern hybridizations, DNA was transferred to nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, N.H.) as described in the manufacturer's directions.

The plasmid pABN1 (7) (see Fig. 2), which contains the aerobactin biosynthesis (*iuc*) and ferric-aerobactin receptor (*iutA*) genes of the pColV-K30 plasmid of *E. coli*, was the source of nucleic acid probes. The probes (see Fig. 2) were as follows: (i) the 7.0-kb *Hind*III-*Eco*RI fragment of pABN1 (*iuc*-*iutA* probe), which contained genes for aerobactin biosynthesis (*iucABCD*) and the truncated gene for the ferric-aerobactin receptor (*iutA*), and (ii) the 1.8-kb *Pvu*II-*Kpn*I fragment (*iutA* gene probe). Neither of the probes contain the *ISI*-like element proximal to the aerobactin operon in pColV-K30 (44) and present on other plasmids and chromosomes of enteric bacteria. Restriction fragments used as probes were purified from agarose gels by adsorption and elution from NA-45 DEAE membranes as described in the recommendations of the manufacturer (Schleicher & Schuell). Nucleic acid probes, prepared by nick translation of isolated fragments with biotin-ATP (Gibco BRL Life Technologies, Inc., Gaithersburg, Md.), were used at concentrations of 0.2 μ g/ml of hybridization solution. Hybridizations were visualized following development with a non-radioactive nucleic acid detection kit (BLUGENE; Gibco BRL Life Technologies). Radioactive probes, labelled by nick translation or random primer extension with [^{32}P]dCTP as described in the recommendations of the manufacturer (Gibco BRL Life Technologies), were used in some experiments. Hybridization conditions were moderately stringent (42°C, 50% formamide, and 0.16 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) and were followed by washes at 55°C in 0.1 $\times$ SSC.

Genomic library construction. Genomic DNA purified from *E. cloacae* 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 Cloning Systems, La Jolla, Calif.); *E. coli* DH5 α was transduced with packaged DNA, and transductants were selected on LB medium with tetracycline. Mobilization of pLAFR3 and derivatives from *E. coli* into *E. cloacae* was accomplished by triparental matings with DH5 α (PRK2013)

(14) as a helper. Transconjugants (*E. cloacae* harboring pLAFR3 derivatives) were selected on LB agar amended with cephalaxin, to which strains of *E. cloacae* were resistant, and tetracycline (60 μ g/ml). Transconjugants of *E. cloacae* EcCT-501 harboring pLAFR3 were obtained typically at a frequency of ca. 10^{-4} transconjugant per recipient.

Cloacin-DF13 sensitivity. The presence of a ferric-aerobactin outer membrane receptor in *E. coli* was demonstrated by measuring sensitivity to cloacin-DF13, a bacteriocin that recognizes the ferric-aerobactin receptor protein (6, 54). Strains were tested for sensitivity to crude preparations of cloacin DF13 obtained from filtrates of cultures of *E. cloacae* DF13, which were treated with mitomycin (1 μ g/ml), as previously described (10). Cultures to be tested for cloacin DF13 sensitivity were grown overnight in TMS and adjusted to a uniform density of 0.1 optical density at 640 nm. Ten microliters of the adjusted suspension was added to 10 ml of nutrient broth (Difco Laboratories) containing 0.5% (wt/vol) molten agar. The molten agar suspension was poured onto 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 35°C for 18 h and observed for clear zones of growth inhibition surrounding the cloacin DF13 preparation.

Derivation of an *luc*⁻ derivative of *E. cloacae*. The marker exchange-eversion mutagenesis technique of Ried and Collier (45) was used to construct directed, unmarked mutants of *E. cloacae* EcCT-501 and its rifampin-resistant derivative, JL1157. The *nptI*-*sacB*-*sacR* cartridge, which is carried on a 3.8-kb *Bam*HI fragment of pUM24 (45), confers kanamycin resistance to *E. cloacae*, because of *nptI*, and sucrose sensitivity, because of the production of levan sucrase by *sacB*. *E. cloacae* grew on 925 agar medium (24), 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 that resulted in insertion of the cartridge into the bacterial genome were selected on LB medium with kanamycin; those resulting in eviction of the cartridge from the genome were selected on 925 agar medium containing 10% sucrose. In preliminary experiments, pLAFR3 was not maintained in *E. cloacae* 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 EcCT-501 could be detected. Cultures of EcCT-501 and its rifampin-resistant derivative JL1157, both containing pLAFR3, were grown at 35°C with shaking in 200 ml of LB broth in the absence of tetracycline. After 10 to 24 h, 0.1 ml of culture was transferred to 200 ml of fresh LB medium. After four or five successive transfers, pLAFR3 was generally lost from greater than 90% of bacterial cells.

Conditions of iron-limited growth. Strains of *E. cloacae* were grown overnight at 35°C with shaking in TMS broth amended with 0.1 μ M FeCl_3 . Three microliters of the culture was spotted onto the surface of TMS agar supplemented with 50, 100, 150, 200, or 250 μ M 2,2'-dipyridyl. Plates were incubated at 35°C and observed 24 and 48 h after inoculation for bacterial growth.

Biological control tests. A 1:1 (vol/vol) mixture of Newberg fine sandy loam and river sand was used for all experiments. The soil, pH 6.0, contained 0.02% total nitrogen, 9 mg of phosphorus per kg, and 32 mg of iron per kg. Raw soil was infested with a dry-soil inoculum of isolate N1 of *P. ultimum*, prepared as described by Paulitz and Baker (40), to obtain 100 propagules per g of soil. Propagules of *P. ultimum* were

enumerated from dry soil inoculum immediately prior to use by spreading aliquots of serial dilutions of soil inoculum onto the surface of Mircetich medium (32). Infested soil was equilibrated to a soil moisture of -0.01 MPa as described previously (23). Bacterial cells from LB agar cultures were dislodged into sterile, deionized water, washed by centrifugation, and resuspended in water to a final concentration of 10^9 CFU/ml. Cucumber seeds (cv. Marketmore) were soaked in bacterial suspensions for 10 to 20 min prior to planting. Each treatment was comprised of 10 replicate plastic cups each containing 300 g of infested soil; three seeds were planted into each cup. As a fungicide treatment for comparison with the bacterial seed treatments, 75 μ g of metalaxyl (Ciba-Geigy, Greensboro, N.C.) was applied as an aqueous drench to the soil surface immediately before planting. Seedling emergence was scored 7 days after planting. Rhizosphere population sizes of the rifampin-resistant strain JL1157 and its *Iuc*⁻ derivative were estimated from entire root systems of 10 replicate plants by dilution plating of root washings on King's Medium B amended with 100 μ g of rifampin per ml and 50 μ g of cycloheximide per ml (27). The experiment was repeated.

Data analysis. Seedling emergence and bacterial rhizosphere population data for bacterial treatments were analyzed statistically by the general linear model procedure of Statistical Analysis Systems (SAS Institute, Inc., Cary, N.C.). To achieve homogeneity of variances, the seedling emergence data from the metalaxyl treatment, which generally was more uniform than that of bacterial treatments, was removed from the data set prior to analysis. The logarithmic (base 10) transformation was applied to individual estimations of rhizosphere bacterial population size prior to statistical analysis.

RESULTS

Survey of catechol and hydroxamate production by strains of *E. cloacae*. Five strains of *E. cloacae* that were isolated from seed or plant surfaces and function as effective agents for the biological control of *Pythium* damping-off diseases (EcCT-501, E1, E6, E13, and EcH-1; Table 1) produced catechol and hydroxamate in culture. Neither catechol nor hydroxamate was produced by strains of *E. cloacae* grown in medium containing 100 μ M FeCl_3 . To determine if the hydroxamate produced by *E. cloacae* was aerobactin, the capacity of hydroxamate-producing strains to cross-feed *E. coli* LG1522 was evaluated. *E. coli* LG1522 does not produce aerobactin or enterobactin, lacks the ferric-enterobactin outer membrane receptor protein, but has the ferric-aerobactin receptor protein (10, 57). Thus, strain LG1522 grows under iron-limiting conditions only if it is provided with ferric-aerobactin. The five strains of *E. cloacae* cross-fed strain LG1522 and produced the large halos on CAS agar that are characteristic of aerobactin-producing strains of *E. coli* (49). Because *E. cloacae* EcCT-501 was a proficient recipient of conjugative plasmids in matings with *E. coli* donors, this strain was selected for detailed molecular and biochemical analysis of its hydroxamate iron acquisition system.

Purification and characterization of aerobactin produced by *E. cloacae*. Aerobactin, purified from *E. coli* LG1315, and the iron-reactive hydroxamate isolated from *E. cloacae* EcCT-501 had in common an R_f of 0.58 on thin-layer chromatography plates. No additional hydroxamates were detected in culture supernatants of *E. cloacae* EcCT-501. The proton nuclear magnetic resonance spectrum of the hydroxamate

TABLE 2. Proton nuclear magnetic resonance chemical shift data for aerobactin in deuterium oxide isolated from *Escherichia coli* LG1315 and from *Enterobacter cloacae* EcCT-501

Proton assignment	Chemical shift (multiplicity) ^a	
	<i>E. coli</i>	<i>E. cloacae</i>
Lys α	4.08 (t)	4.09 (t)
Lys ϵ	3.54 (t)	3.53 (t)
Citrate CH_2	2.67 (d of q)	2.65 (d of q)
Acetyl CH_3	2.06 (s)	1.99–2.02 (s)
Lys β , δ	1.48–1.83 (m)	1.55–1.75 (m)
Lys γ	1.22–1.38 (m)	1.24–1.28 (m)

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

from *E. cloacae* was consistent with that of authentic aerobactin (Table 2) and with the published spectrum of aerobactin (15).

Identification and cloning of aerobactin biosynthesis genes of *E. cloacae* EcCT-501. To identify aerobactin biosynthesis genes of *E. cloacae*, we screened individual members of a genomic library of EcCT-501 for aerobactin production. Six of 1,066 cosmids that composed the genomic library of EcCT-501 conferred aerobactin production to *E. coli* DH5 α , as indicated by hydroxamate production, cross-feeding of *E. coli* LG1522, and production of large orange halos on CAS agar. Cells of *E. coli* harboring any of the six cosmids also were sensitive to cloacin DF13, indicating that the cloned region encoded the outer membrane protein that is a receptor for both ferric-aerobactin and cloacin-DF13. The six cosmids contained a common region of 12.3 kb that hybridized to genes for aerobactin biosynthesis (*iuc* for iron uptake chelate) and the ferric-aerobactin receptor protein (*iutA* for iron uptake transport) of *E. coli* (Fig. 1). A single 5.5-kb *EcoRI* fragment in the cloned aerobactin region of *E. cloacae* hybridized to the *iutA* gene probe. Comparison of restriction maps of aerobactin production and receptor regions revealed substantial differences between those present on the pColV-K30 plasmid of *E. coli* (8) or *E. cloacae* EcCT-501 (Fig. 2).

Derivation of an *Iuc*⁻ mutant of *E. cloacae*. Sequences within the *iuc* region were deleted from the genome of *E. cloacae* by the marker-exchange-eviction mutagenesis technique of Ried and Collmer (45). The *nptI-sacB-sacR* cartridge was cloned into the unique *BglII* site of the 4.4-kb *EcoRI* fragment that contained a portion of the *iuc* region of EcCT-501 (Fig. 3). The *nptI-sacB-sacR* cartridge and flanking sequences then were cloned into the unique *EcoRI* site of pLAFR3 to construct pJEL1741 (Fig. 3). The two *SstI* fragments internal to pJEL1574 were deleted to construct pJEL1771, an unstable plasmid with a deletion in the *iuc* region (Fig. 3). The cartridge-containing *iuc* region of pJEL1741 was exchanged into wild-type strain EcCT-501 to produce LA115. The cartridge was then evicted from LA115 by exchange substitution of the sequences in pJEL1771 to produce LA121. An *Iuc*⁻ mutant of JL1157, a rifampin-resistant derivative of strain EcCT-501, was similarly derived by exchange substitution with regions cloned in pJEL1741 and pJEL1771. Southern analysis of restriction enzyme-digested DNA probed with biotin-labelled pJEL1741 confirmed that insertions and deletions into the genomic DNA of EcCT-501 and JL1157 were the same as those illustrated in Fig. 3 (data not shown).

Characterization of an *Iuc*⁻ derivative of *E. cloacae*. Strain LA121, which contained a genomic deletion in the *iuc*

FIG. 1. Restriction maps of cosmids containing aerobactin biosynthesis and uptake genes from *E. cloacae* EcCT-501. Restriction fragments that hybridized to *iuc-iutA* or *iutA* probes (shown in Fig. 2) are indicated by thick bars below the cosmid maps. The *EcoRI* (E) and *HindIII* (H) restriction sites shown on the extreme right and left of the inserts were from the polylinker of pLAFR3. In cosmid pJEL1582, the *EcoRI* site of the polylinker mapped very closely to an *EcoRI* site in the cloned genomic DNA of EcCT-501.

region, did not produce aerobactin but produced catechol and grew at a rate comparable to that of parental strain EcCT-501 in LB medium (Table 3). Cosmid pJEL1574, which contained the cloned *iuc* and *iut* genes of EcCT-501 (Fig. 1), restored aerobactin production to LA121. Therefore, a role of the cloned region in aerobactin production of strain EcCT-501 was confirmed. Both *Iuc*⁺ and *Iuc*⁻ strains grew on an iron-deficient medium (TMS containing up to 250 μ M 2,2'-dipyridyl); thus, a role for aerobactin in iron acqui-

sition by *E. cloacae* was not demonstrated in this study. It is possible that a catechol siderophore iron acquisition system present in both *Iuc*⁺ and *Iuc*⁻ strains enabled bacterial growth irrespective of a functional aerobactin system.

Biological control of *Pythium* damping-off of cucumber. Treatment of seed with EcCT-501 or the *Iuc*⁻ derivative LA121 significantly increased seedling emergence from soil infested with *P. ultimum* (Table 3). Similarly, the rifampin-resistant strain JL1157 and its *Iuc*⁻ derivative LA122 effec-

FIG. 2. Restriction maps of aerobactin regions from *E. cloacae* EcCT-501 or from the pColV-K30 plasmid of *E. coli* (7). The aerobactin region of EcCT-501 corresponds to the three *EcoRI* fragments of pJEL1584 that hybridized to the *iuc-iutA* probe, as shown in Fig. 1. The *EcoRI* site on the extreme left was contributed by the polylinker of pLAFR3. Restriction fragments of pColV-K30 aerobactin regions used as probes are denoted as thick, shaded (*iuc-iutA*) or solid (*iutA*) bars.

FIG. 3. Restriction maps of insertion and deletion derivatives of the cloned aerobactin region used to construct the *luc*⁻ mutant of *E. cloacae*. Dashed lines denote the insertion of the *nptI-sacB-sacR* cartridge into the unique *Bgl*II site of the subcloned 4.4-kb *Eco*RI fragment from pJEL1574 to create pJEL1741. Plasmid pJEL1771 was derived from pJEL1574 by deletion of two internal *Sst*I fragments. Plasmids were used to mutate strain EcCT-501 to LA121 (*luc*⁻) via a series of gene replacements: pJEL1741 (EcCT-501 to LA115) and pJEL1771 (LA115 to LA121).

tively controlled preemergence damping-off of cucumber caused by *P. ultimum* (data not shown). The biocontrol activities of *luc*⁻ derivatives did not differ significantly from *luc*⁺ parental strains. The mean rhizosphere population sizes established by JL1157 and its *luc*⁻ derivative LA122 did not differ significantly; both established rhizosphere population sizes of 6.0 log(CFU per root system). Thus, aerobactin production did not contribute significantly to the biological control activity or rhizosphere establishment of *E. cloacae* in this study.

DISCUSSION

Five plant-associated strains of *E. cloacae* produced a catechol(s), a hydroxamate(s), and a siderophore(s), as determined on CAS agar, a universal siderophore detection medium (49). On the basis of specific cross-feeding of ferric-aerobactin-utilizing strains of *E. coli*, we suggest that aerobactin was produced by the five strains of *E. cloacae*. Thus, in contrast to 11 plant-associated strains of *Enterobacter*

aerogenes, which do not produce aerobactin (17), certain plant-associated strains of *E. cloacae* produced this siderophore. Nevertheless, aerobactin production is not a ubiquitous phenotype of plant-associated strains of *E. cloacae*; a study recently completed in our laboratory demonstrated that *E. cloacae* OK-1, which was isolated from the rhizosphere of okra (33a), did not produce aerobactin (10a). The present study focused on aerobactin production by *E. cloacae* EcCT-501, which was demonstrated by several lines of evidence. The proton nuclear magnetic resonance spectrum of the sole hydroxamate produced by strain EcCT-501 was identical to that of authentic aerobactin (Table 2) and to the published spectrum of aerobactin (15). Hybridization experiments with aerobactin-specific probes from the pColV-K30 plasmid of *E. coli* demonstrated the presence of aerobactin biosynthesis and ferric-aerobactin uptake genes in *E. cloacae* (Fig. 1). Cloned genes encoding the aerobactin-iron uptake system of *E. cloacae* conferred aerobactin production and cloacin-DF13 sensitivity to host cells of *E. coli*. We propose that genes encoding the aerobactin iron

TABLE 3. Characteristics of an *luc*⁻ derivative of *E. cloacae* EcCT-501

Strain	Phenotype ^a	Hydroxamate production ^b		Catechol production ^c		Generation time (min)	% Emergence of cucumber seedlings ^d
		0.1 μ M FeCl ₃	100.0 μ M FeCl ₃	0.1 μ M FeCl ₃	100.0 μ M FeCl ₃		
EcCT-501	<i>luc</i> ⁺	350	4	37	1	31	87a
LA121	<i>luc</i> ⁻	7	4	30	1	32	77a
LA121(pJEL1574)	<i>luc</i> ⁺	420	7	44	4	NT	NT

^a *luc*⁺, aerobactin production based on cross-feeding of aerobactin indicator strain *E. coli* LG1522 and characteristic halos surrounding colonies grown on CAS agar (49); *luc*⁻, no aerobactin production.

^b The micromolar equivalents of hydroxamate produced by cultures grown at two FeCl₃ concentrations were assayed by the method of Csáky (12). Values are the average of three replicate cultures, in which the micromolar concentration of hydroxamate is adjusted for cell density (A_{600}) of each culture.

^c The micromolar equivalents of catechol produced by cultures grown at two FeCl₃ concentrations were assayed by the method of Rioux et al. (46). Values are the average of three replicate cultures, in which the micromolar concentration of catechol is adjusted for cell density (A_{600}) of each culture.

^d Mean seedling emergence from cucumber seed treated with bacterial strains and planted in soil infested with *P. ultimum* is presented. Values followed by a common letter do not differ significantly ($P = 0.05$) as determined by Duncan's multiple-range test. Mean percent emergence from nontreated and metalaxyl-treated seed was 40b and 93, respectively. NT, not tested.

acquisition system are located on the chromosome because no plasmid could be isolated from *E. cloacae* EcCT-501. Nevertheless, the possibility remains that an unidentified plasmid, which was not detected by the standard plasmid extraction procedures used in this study, may encode for aerobactin biosynthesis in *E. cloacae* EcCT-501.

Aerobactin-mediated iron uptake systems are present in many genera of the family Enterobacteriaceae, and yet the genetic determinants of aerobactin biosynthesis and uptake vary among these bacteria (42). Aerobactin biosynthesis genes of plasmid or chromosomal origin appear highly conserved among *E. coli* and *Shigella* spp. (8, 25, 29, 30, 53). In contrast, aerobactin biosynthesis genes of clinical isolates of *E. cloacae* do not hybridize to those of pColV-K30 (11). Aerobactin biosynthesis genes of *E. cloacae* EcCT-501 hybridized to those of pColV-K30, but restriction maps of aerobactin regions of the two species differed (Fig. 2). The restriction maps of aerobactin genes from *E. carotovora* (21), *A. aerogenes* (55), and *E. cloacae* (11) also differ from the map proposed here for *E. cloacae* EcCT-501 (Fig. 2). The restriction maps of aerobactin genes of the environmental isolates of *E. cloacae* evaluated in this study also differed from one another. Southern analysis of genomic DNA of four aerobactin-producing strains of *E. cloacae* (E6, E13, E1, and EcH-1; Table 1) revealed two distinct patterns of restriction fragments that hybridized to the *iutA* probe (20a), both of which differed from the restriction map of the aerobactin region of strain EcCT-501. Thus, the results of this study provide further evidence for the heterogeneity of aerobactin genes among clinical and environmental strains of the Enterobacteriaceae.

Molecular approaches have been invaluable in elucidating mechanisms involved in the biological control of plant disease. Nevertheless, molecular analysis of biological control activity has been directed primarily towards antagonistic strains of *Pseudomonas* spp. or *Agrobacterium radiobacter*. In this study, we developed a system for the genetic analysis of a strain of *E. cloacae* that suppressed *Pythium* damping-off diseases. The genomic library constructed here coupled with the transposon mutagenesis system described by Nelson and Maloney (37) provide powerful tools for the identification of phenotypes that are critical to the biological control activity and ecological fitness of *E. cloacae*.

Aerobactin production was not essential for biological control activity of *E. cloacae* under the conditions of this study: an aerobactin-deficient mutant (*luc*⁻) of *E. cloacae* EcCT-501 was as effective as the parental strain in suppression of *Pythium* damping-off of cucumber. Nevertheless, aerobactin production may contribute to the suppression by *E. cloacae* of *Pythium* damping-off under other environmental conditions or on plant hosts other than cucumber. For example, pyoverdine siderophores produced by *Pseudomonas* spp. contribute significantly to biological control of *Pythium* damping-off of cotton by *P. fluorescens* (27) but have no demonstrated role in biocontrol of *Pythium* damping-off of cucumber by strains of *P. putida* (41) or *P. fluorescens* (23). Possible explanations for the variable role of pyoverdine siderophores in biological control of *Pythium* damping-off include variation in composition or quantity of seed exudates, soil type and temperature, *Pseudomonas* species and strain, isolate of *P. ultimum*, and the duration observed between seed planting and seedling emergence (3 to 5 days for cucumber and 7 to 14 days for cotton) (41). Although aerobactin production may contribute to the biological control activity of *E. cloacae* on certain plant hosts or under certain environmental conditions, unidentified pheno-

types common to strain EcCT-501 and *luc*⁻ derivatives apparently determined their activities against *Pythium* damping-off of cucumber in this study. This finding does not exclude the possibility that siderophore-mediated iron competition contributes to biological control of *Pythium* damping-off by *E. cloacae*. *luc*⁻ mutants of EcCT-501 produce a catechol siderophore(s), which may contribute to ecological fitness, iron competition with other microorganisms, and biological control activity. Future studies evaluating the rhizosphere population sizes and relative biological control activities of *E. cloacae* EcCT-501 and derivatives deficient in both aerobactin and catechol siderophore production will provide a greater insight into the role of siderophores in the ecology and antagonistic properties of this organism.

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REFERENCES

1. Arnow, L. E. 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. *J. Biol. Chem.* **118**:531-537.
2. Atkin, C. L., J. B. Neilands, and H. J. Phaff. 1970. Rhodotorulic acid from species of *Leucosporidium*, *Rhodospiridium*, *Rhodotorula*, *Sporidiobolus*, and *Sporobolomyces*, and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*. *J. Bacteriol.* **103**:722-733.
3. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl. 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
4. Becker, J. O., and R. J. Cook. 1988. Role of siderophores in suppression of *Pythium* species and production of increased-growth response of wheat by fluorescent pseudomonads. *Phytopathology* **78**:778-782.
5. Bell, S. J., S. A. Friedman, and J. Leong. 1979. Antibiotic action of *N*-methylthioformohydroxamate metal complexes. *Antimicrob. Agents Chemother.* **15**:384-391.
6. Bindereif, A., V. Braun, and K. Hantke. 1982. The cloacin receptor of ColV-bearing *Escherichia coli* is part of the Fe³⁺-aerobactin transport system. *J. Bacteriol.* **150**:1472-1475.
7. Bindereif, A., and J. B. Neilands. 1983. Cloning of the aerobactin-mediated iron assimilation system of plasmid ColV. *J. Bacteriol.* **153**:1111-1113.
8. Bindereif, A., and J. B. Neilands. 1985. Aerobactin genes in clinical isolates of *Escherichia coli*. *J. Bacteriol.* **161**:727-735.
9. Brenner, D. J. 1981. The genus *Enterobacter*, p. 1173-1180. In M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), *The prokaryotes: a handbook on habitats, isolation, and identification of bacteria*, vol. 2. Springer-Verlag, Berlin.
10. Carbonetti, N. H., and P. H. Williams. 1984. A cluster of five genes specifying the aerobactin iron uptake system of plasmid ColV-K30. *Infect. Immun.* **46**:7-12.
- 10a. Carnegie, S. R., and J. E. Loper. Unpublished data.
11. Crosa, L. M., M. K. Wolf, L. A. Actis, J. Sanders-Loehr, and J. H. Crosa. 1988. New aerobactin-mediated iron uptake system in a septicemia-causing strain of *Enterobacter cloacae*. *J. Bacteriol.* **170**:5539-5544.
12. Csáky, T. Z. 1948. On the estimation of bound hydroxylamine in biological materials. *Acta Chem. Scand.* **2**:450-454.
13. Elad, Y., and I. Chet. 1987. Possible role of competition for nutrients in biocontrol of *Pythium* damping-off by bacteria.

- Phytopathology 77:190-195.
14. Figurski, D. H., and D. R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA 76:1648-1652.
 15. Gibson, F., and D. I. Magrath. 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes* 62-1. Biochem. Biophys. Acta 192:175-184.
 16. Gutterson, N. 1990. Microbial fungicides: recent approaches to elucidating mechanisms. Crit. Rev. Biotechnol. 10:69-91.
 17. Haahtela, K., R. Rönkkö, T. Laakso, P. H. Williams, and T. K. Korhonen. 1990. Root-associated *Enterobacter* and *Klebsiella* in *Poa pratensis*: characterization of an iron-scavenging system and a substance stimulating root hair production. Mol. Plant-Microbe Interact. 3:358-365.
 18. Howell, C. R., R. C. Beier, and R. D. Stipanovic. 1988. Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of *Pythium* preemergence damping-off by the bacterium. Phytopathology 78:1075-1078.
 19. Howell, C. R., and R. D. Stipanovic. 1980. Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic pyoluteorin. Phytopathology 70:712-715.
 20. Howie, J. H., and T. V. Suslow. 1991. Role of antibiotic biosynthesis in the inhibition of *Pythium ultimum* in the cotton spermosphere and rhizosphere by *Pseudomonas fluorescens*. Mol. Plant-Microbe Interact. 4:393-399.
 - 20a. Ishimaru, C. A. Unpublished data.
 21. Ishimaru, C. A., and J. E. Loper. 1992. High-affinity iron uptake systems present in *Erwinia carotovora* subsp. *carotovora* include the hydroxamate siderophore aerobactin. J. Bacteriol. 174:2993-3003.
 22. Ishimaru, C. A., A. Vanavichit, and J. E. Loper. 1989. Genetic analysis of the aerobactin iron uptake system of an *Enterobacter cloacae* strain antagonistic against *Pythium* spp. Phytopathology 79:909. (Abstract.)
 23. Kraus, J., and J. E. Loper. 1992. Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of *Pythium* damping-off of cucumber. Phytopathology 82:264-271.
 24. Langley, R. A., and C. I. Kado. 1972. Studies on *Agrobacterium tumefaciens*. Conditions for mutagenesis of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and relationships of *A. tumefaciens* mutants to crown-gall tumor induction. Mutat. Res. 14:277-286.
 25. Lawlor, K. M., and S. M. Payne. 1984. Aerobactin genes in *Shigella* spp. J. Bacteriol. 160:266-272.
 26. Leong, J. 1986. Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. Annu. Rev. Phytopathol. 24:187-209.
 27. Loper, J. E. 1988. Role of fluorescent siderophore production in biological control of *Pythium ultimum* by a *Pseudomonas fluorescens* strain. Phytopathology 78:166-172.
 28. Loper, J. E., and J. S. Buyer. 1991. Siderophores in microbial interactions on plant surfaces. Mol. Plant-Microbe Interact. 4:5-13.
 29. Marolda, C. L., M. A. Valvano, K. M. Lawlor, S. M. Payne, and J. H. Crosa. 1987. Flanking and internal regions of chromosomal genes mediating aerobactin iron uptake systems in enteroinvasive *Escherichia coli* and *Shigella flexneri*. J. Gen. Microbiol. 133:2269-2278.
 30. McDougall, S., and J. B. Neilands. 1984. Plasmid- and chromosome-coded aerobactin synthesis in enteric bacteria: insertion sequences flank operon in plasmid-mediated systems. J. Bacteriol. 159:300-305.
 31. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 32. Mircetich, S. M., and J. M. Kraft. 1973. Efficiency of various selective media in determining *Pythium* populations in soil. Mycopathol. Mycol. Appl. 50:151-161.
 33. Neilands, J. B. 1981. Microbial iron compounds. Annu. Rev. Biochem. 50:715-731.
 - 33a. Nelson, E. B. Personal communication.
 34. Nelson, E. B. 1988. Biological control of *Pythium* seed rot and preemergence damping-off of cotton with *Enterobacter cloacae* and *Erwinia herbicola* applied as seed treatments. Plant Dis. 72:140-142.
 35. Nelson, E. B. 1990. Exudate molecules initiating fungal responses to seeds and roots. Plant Soil 129:61-73.
 36. Nelson, E. B., and C. M. Craft. 1991. Introduction and establishment of strains of *Enterobacter cloacae* in golf course turf for the biological control of dollar spot. Plant Dis. 75:510-514.
 37. Nelson, E. B., and A. P. Maloney. 1992. Molecular approaches for understanding biological control mechanisms in bacteria: studies of the interaction of *Enterobacter cloacae* with *Pythium ultimum*. Can. J. Plant Pathol. 14:106-114.
 38. Nelson, E. B., W.-L. Chao, J. M. Norton, G. T. Nash, and G. E. Harman. 1986. Attachment of *Enterobacter cloacae* to hyphae of *Pythium ultimum*: possible role in the biological control of *Pythium* preemergence damping-off. Phytopathology 76:327-335.
 39. Osburn, R. M., M. N. Schroth, J. G. Hancock, and M. Henderson. 1989. Dynamics of sugarbeet seed colonization by *Pythium ultimum* and *Pseudomonas* species: effect on seed rot and damping-off. Phytopathology 79:709-716.
 40. Paulitz, T. C., and R. Baker. 1987. Biological control of *Pythium* damping-off of cucumbers with *Pythium nunn*: population dynamics and disease suppression. Phytopathology 77:335-340.
 41. Paulitz, T. C., and J. E. Loper. 1991. Lack of a role for fluorescent siderophore production in the biological control of *Pythium* damping-off of cucumber by a strain of *Pseudomonas putida*. Phytopathology 81:930-935.
 42. Payne, S. M. 1980. Synthesis and utilization of siderophores by *Shigella flexneri*. J. Bacteriol. 143:1420-1424.
 43. Payne, S. M. 1988. Iron and virulence in the family Enterobacteriaceae. Crit. Rev. Microbiol. 16:81-111.
 44. Perez-Casal, J. F., and J. H. Crosa. 1984. Aerobactin iron uptake sequences in plasmid ColV-K30 are flanked by inverted IS₁-like elements and replication regions. J. Bacteriol. 160:256-265.
 45. Ried, J. L., and A. Collmer. 1987. An *nptI-sacB-sacR* cartridge for constructing directed, unmarked mutations in Gram-negative bacteria by marker exchange-eviction mutagenesis. Gene 57:239-246.
 46. Rioux, C., D. C. Jordan, and J. B. M. Rattray. 1983. Colorimetric determination of catechol siderophores in microbial cultures. Anal. Biochem. 133:163-169.
 47. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 48. Schippers, B., A. W. Bakker, and P. A. H. M. Bakker. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Annu. Rev. Phytopathol. 25:339-358.
 49. Schwyn, B., and J. B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160:47-56.
 50. Simon, E. H., and I. Tessman. 1963. Thymidine-requiring mutants of phage T4. Proc. Natl. Acad. Sci. USA 50:526-532.
 51. Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.
 52. Trutmann, P., and E. Nelson. 1992. Production of non-volatile and volatile inhibitors of *Pythium ultimum* sporangium germination and mycelial growth by strains of *Enterobacter cloacae*. Phytopathology 82:1120. (Abstract.)
 53. Valvano, M. A., and J. H. Crosa. 1984. Aerobactin iron transport genes commonly encoded by certain ColV plasmids occur in the chromosome of a human invasive strain of *Escherichia coli* K1. Infect. Immun. 46:159-167.
 54. Van Tiel-Menkveld, G. J., J. M. Mentjox-Vervuurt, B. Oudega, and F. K. de Graaf. 1982. Siderophore production by *Enterobacter cloacae* and a common receptor protein for the uptake of aerobactin and cloacin DF13. J. Bacteriol. 150:490-497.

55. **Waters, V. L., and J. H. Crosa.** 1988. Divergence of the aerobactin iron uptake systems encoded by plasmids pColV-K30 in *Escherichia coli* K-12 and pSMN1 in *Aerobacter aerogenes* 62-1. *J. Bacteriol.* **170**:5153-5160.
56. **Williams, P. H.** 1979. Novel iron uptake system specified by ColV plasmids: an important component in the virulence of invasive strains of *Escherichia coli*. *Infect. Immun.* **26**:925-932.
57. **Williams, P. H., and P. J. Warner.** 1980. ColV plasmid-mediated, colicin V-independent iron uptake system of invasive strains of *Escherichia coli*. *Infect. Immun.* **29**:411-416.
58. **Wuthrich, K.** 1986. NMR of proteins and nucleic acids. John Wiley & Sons, Inc., New York.